THE IMPORTANCE OF DORMANT EGG BANK DYNAMICS IN ECOTOXICOLOGICAL EFFECT ASSESSMENT:

From laboratory to mesocosm studies

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Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in Science (Biology)

August 2015

This research was financially supported by the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen)

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ACKNOWLEDGEMENTS

Er zijn een aantal mensen die een hele dikke “DANKJEWEL” verdienen voor hun hulp en steun in de afgelopen 4,5 jaar. Ik zal starten met de persoon zonder wie dit doctoraat (letterlijk) nooit mogelijk zou zijn geweest: mijn promotor Luc Brendonck. Na bijna vier jaar in de industrie gewerkt te hebben wist ik dat het tijd was voor iets nieuws. Luc, ik ben toen bij u gekomen om te polsen naar de mogelijkheden voor een doctoraat. Ondanks dat ik een totale outsider was op het labo (zowel qua achtergrond als qua afkomst), hebt u mij vanaf het begin gesteund en na samen een projectvoorstel uitgedacht te hebben, door alle IWT hindernissen heen geloodst. Alleen daarvoor al mijn eeuwige dank! Bedankt ook dat ik op uw tijd en begeleiding kon blijven rekenen de afgelopen jaren, tot op het allerlaatste moment.

Aline, misschien niet van in het prille begin, maar zeker wel tot het bittere einde (zelfs tot in de Brenne)… enorm hard bedankt voor al je hulp bij het uitdenken van experimenten, praktische adviezen op de Arena en in het labo, je kritisch oog voor het maken van figuren, ideeën voor beter gestructureerde teksten en vooral de fijne samenwerking! Als ik ergens mee vast zat, was je nuchtere en analytische kijk op de wereld vaak al voldoende om een oplossing of alternatief te vinden. Merci!

Luc DM, bedankt om gaatjes vrij te maken in uw overvolle planning, om mee na te denken over het mesocosm experiment en het helpen verbeteren van mijn manuscripten. Uw handschrift kan ik ondertussen al bijna ontcijferen…

Ik zou graag ook de leden van mijn doctoraatsjury (Mieke Jansen, Robby Stoks, Erik Smolders, Colin Janssen en David Angeler) willen bedanken voor de goede feedback tijdens en op het einde van mijn doctoraat. Bedankt voor de discussies en nuttige suggesties om de thesis te verbeteren. David, a special thanks for making time to evaluate my PhD and coming all the way from Sweden to Leuven for attending the public defense.

En natuurlijk, de Brennies: allemaal bedankt voor jullie gezelschap op het labo, tijdens de jaarlijkse brennie-bbq´s en lasershootings! Ik heb met veel plezier vier jaar in het meest drukke, chaotische en gezellige bureau van het labo doorgebracht. Tom (bedankt voor je grenzeloos optimisme en behulpzaamheid, zelfs al tijdens mijn IWT aanvraag), Jane (merci voor de decoratie van mijn bureau, en het altijd wisselende uitzicht achter mijn pc-scherm), team Killi (ik ga jullie humor en enthousiasme missen, en Arnout “the Wall”, bedankt voor alle koffie, koekjes en chocolade van het winkeltje), Karen (onze uitstapjes naar Gent, Antwerpen en de Black Keys zal ik niet snel vergeten). En wat verder weg, maar daarom niet minder belangrijk, de rest van de Brennies: Maarten, Daan, Trevor, Semba en Grite (bedankt!). Falko, a big thanks for your support even from South-Africa. Cristina, Irene, even though your stay at the lab was quite short, thanks for your company also after working hours, and hope to see you again soon! En Bram, ik ga u toch moeten bedanken (zowel voor je statistiek advies, als voor de hulp tijdens een zeer zonnige dag op de Arena).

Veel dank ook aan alle (bachelor-, master- en job-) studenten, voor jullie inzet en het vele praktisch werk: Tom Voet, Thomas Nicolaï, Laetitia Beullens, Maarten Goedseels, Camille De Raedemaeker, Ruben Cardoen en Sarah Tilkin.

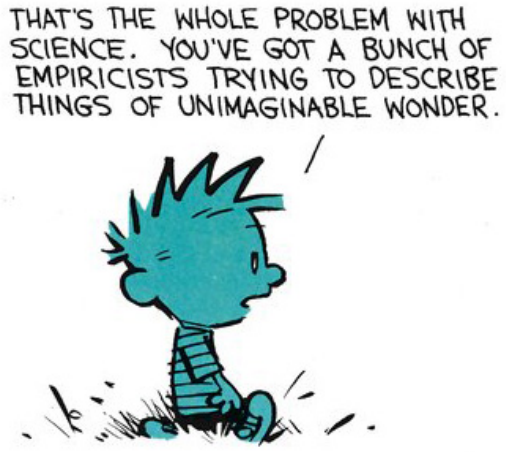
En dan, de rest van het labo… Allereerst, bedankt aan alle ATP´ers voor de nodige ondersteuning! Conny (zonder twijfel de meest efficiënte persoon van het labo, heel erg bedankt voor alle administratieve hulp), Melissa (merci voor alle zonnige, maar vooral ook regenachtige Arena-dagen, met vettige tonnen, Jip en Janneke, dinokoeken en lunch in Alma-3), Ria (voor alle pesticidenvragen en bestellingen) en natuurlijk Rony en Geert (bedankt voor hulp bij de staalname en het vervoeren van al die emmers sediment, het maken van grote en kleine zeven, telbakjes en al de rest). Alle mensen van de Daphnia-groep, die mij als buitenstaander hebben getolereerd in jullie lokalen: bedankt voor het gezelschap in de kelder tijdens experimenten. Mieke, bedankt voor je hulp en de praktische organisatie, zodat er toch altijd ergens plaats was voor al mijn potten en platen. Joost, merci voor je statistiek hulp bij de laatste analyses van de mesocosm dataset (op een qwerty-laptop nog wel). De rest van de collega´s in het Kolenmuseum, bedankt voor jullie gezelschap en alle (wetenschappelijke en minder wetenschappelijke) gesprekken van de afgelopen jaren!

En dan nog een aantal mensen die zelfs van de kelder (van de kelder) een aangename werkomgeving hebben kunnen maken: Adinda (geen idee hoe ik u ooit kan bedanken voor de ontelbare hoeveelheid uitgepikte ephippia, gedecapsuleerde eitjes en gevulde multiwell-platen, niet te vergeten uw zangtalent en West-Vlaamse woordjes), Aurora (uw audioboeken en podcasts waren het beste idee ooit om late avonden en lange weekends op het labo door te komen), en Ine (eigenlijk al een tijdje niet meer op het labo, maar toch bijna mijn derde co-promotor, bedankt voor je hulp bij het ontcijferen van onleesbare geschriften, inspiratie bij het bedenken van titels en berekenen van populatiegroeisnelheden). Daarnaast zijn jullie ook bedankt voor alle toffe niet-labo activiteiten (Jane, jij ook)! Ik hoop dat er nog veel spelletjesavonden en cava Fridays mogen volgen. En Ine, merci ook om mijn yoga-buddy te zijn!

Ook buiten het labo zijn er een aantal mensen die ik graag zou willen bedanken. Stijn, Pieter, Kurt en de rest van de jongetjes: jullie zijn de eerste mensen die ik hier in Leuven heb leren kennen, en ook één van de redenen dat ik hier nog altijd graag woon. De jaren aan de Tervuursevest zal ik nooit vergeten! En Stijn, wie had er ooit gedacht dat we nog altijd zouden gaan lopen? Merci ook Andrea en Kirsten voor alle sportieve kilometers! Evelien, helaas klimmen we allebei niet meer, maar ik ben blij dat we die avonden in de Hungaria ondertussen hebben vervangen door andere, wat meer relaxte activiteiten. Dat was de afgelopen jaren de ideale manier om alle doctoraatsstress even te vergeten, merci daarvoor! Anne-Catherine, bedankt voor alle geocache en fotografie uitstapjes, binnenkort maar eens op zoek gaan naar caches in de VS! Ook bedankt aan alle wandelvriendjes voor de mooie reizen en weekends, ver weg of gewoon in de Ardennen. Ik kijk al enorm uit naar onze trip naar IJsland! Johan, bedankt om vorig jaar samen mee te doen aan de dodentocht, een mooie uitdaging, maar zot zijn doet wel zeer! En dan nog mijn beste vriendinnen in Nederland, ik heb jullie de afgelopen jaren (veel te) weinig gezien: Annemieke (binnenkort nog eens, in de file, naar Oberhausen?), Sabine (samen shoppen verveelt nooit!). And Brigitte, we will meet again in Rome this autumn!

En bijna als laatste, wil ik mijn familie heel erg bedanken. Mirjam en Niek (en natuurlijk ook Sven en Luna), bedankt voor alle gezellige weekendjes in Leuven en Dedemsvaart. Pa en ma, bedankt voor alle vrijheid en onvoorwaardelijke steun die jullie mij altijd gegeven hebben om mijn eigen keuzes te maken. Wie had er ooit gedacht dat dat zou leiden tot een doctoraat in Leuven? Ik in elk geval zeker niet.

Als allerlaatste wil ik mijn nieuwe collega´s bij ARCHE bedanken. Het was niet altijd gemakkelijk om de laatste fase van mijn doctoraat te combineren met een nieuwe job. An, Koen en Federica: heel erg bedankt voor jullie vriendelijkheid, geduld en flexibiliteit tijdens mijn eerste maanden. Tot in september!



GENERAL INTRODUCTION

1. Introduction

Currently, agricultural land comprises about 40% of the worlds land surface, producing food for six billion people (Godfray et al., 2010). Projections are that the global population will continue to increase and a doubling is expected by 2050 (Foley et al., 2011). This will be accompanied by a sharp increase in food demand, putting an even greater pressure on the agricultural sector to increase crop yields than today (Enserink et al., 2013). Since global productive arable areas are limited, fertilizers, pesticides and new crop strains have been increasingly used to boost crop production (Tilman et al., 2002). Unfortunately, this has also resulted in serious environmental impacts, such as elevated nutrient levels, habitat degradation and water pollution (Rockstrom et al., 2009). Contamination of surface waters has become a serious problem in many industrialized and agricultural regions (Schwarzenbach et al., 2006). A recent meta-analysis (Stehle and Schulz, 2015) indicates that agricultural pesticide use poses a larger threat to aquatic biodiversity than previously expected. On a global scale more than 50% of insecticide levels in surface waters were found to exceed regulatory threshold levels.

In the European Union alone, around 280 million kilos of pesticides are used annually (sales of active ingredients 2010; ECPA, 2011). By spray drift, run-off and leaching, a fraction of these pesticides ends up in aquatic water bodies in or surrounding agricultural areas (Fig. 1), thereby potentially affecting also non-target organisms (e.g. Lahr et al., 2000; De Schampheleire et al., 2007; Maltby and Hills, 2008). A better understanding of the effects of pesticides under natural conditions is therefore much needed.

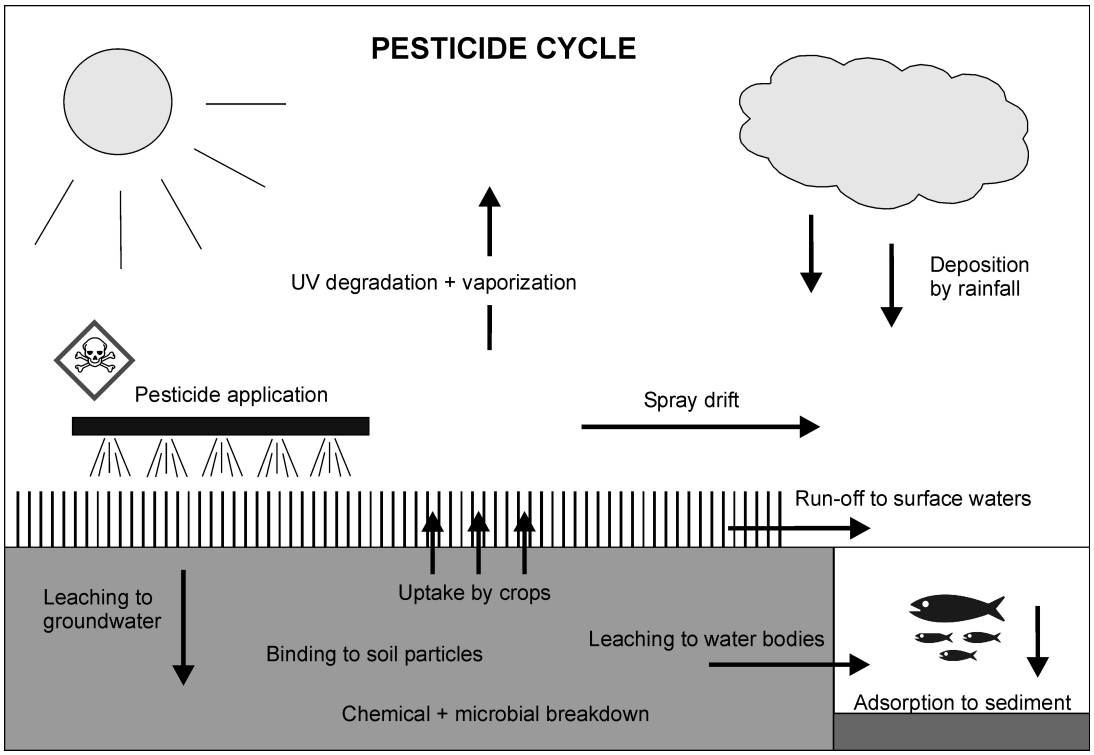


Fig. 1 The pesticide cycle (adapted from R. Cooke, 2000), including various pathways by which pesticides can end up in surface waters.

2. Permanent ponds and shallow lakes

Ponds and small lakes are often located in agricultural areas, where these small-scale elements are important contributors to regional biodiversity (Søndergaard et al., 2005). Individual ponds can support a wide variety of ecological communities. In fact, they can contain more unique and scarce species than other water types, such as rivers and ditches (Williams et al., 2004; Jeffries, 2005). In addition, they have socio-economical value by providing water for cattle, recreation and industrial use (Brönmark and Hansson, 2002) and they are of ecological importance as stepping stones and corridors for biota (De Meester et al., 2005). However, conservation of ponds and shallow lakes has until recently often been neglected (Oertli et al., 2005; De Bie et al., 2008). In many regions, these small water bodies are threatened by anthropogenic disturbances, such as eutrophication, invasion by exotic species and pollution (Boothby, 2003; Curado et al., 2011). Moreover, the number of ponds has been decreasing. It is estimated that about 50% of the ponds in Europe have disappeared over the past century (Boothby and Hull, 1997; Oertli et al., 2005).

Not only are ponds and shallow lakes of ecological importance, they can also serve as excellent model systems for ecological and ecotoxicological research, since they are still quite abundant, span a wide range of ecological gradients, can reflect changes at a larger scale and are relatively easy to sample (De Meester et al., 2005). In addition, pond ecosystems can be quite well represented in artificial ecosystems (micro- and mesocosms), allowing the testing of specific hypothesis, under semi-natural, controlled conditions (De Meester et al., 2005; Van Wijngaarden et al., 2005; De Jong et al., 2008). Zooplankton species living in these small aquatic systems form an integral part of the ecosystem, as the intermediate level between the primary producers (algae) and the secondary consumers (fish) (Miner et al., 2012).

3. Dormant egg bank dynamics

Many zooplankton taxa living in permanent and temporary standing waters reproduce asexually during the growing season, but switch to sexual reproduction when conditions deteriorate (Brendonck and De Meester, 2003; Decaestecker et al., 2009). They produce dormant stages to survive unfavourable environmental conditions, such as drought, low oxygen concentrations, food limitation, crowding and the presence of predators (Hairston et al., 1990; Alekseev and Starobogatov, 1996; Brendonck et al., 1998; Slusarczyk et al., 2005; Fig. 2).

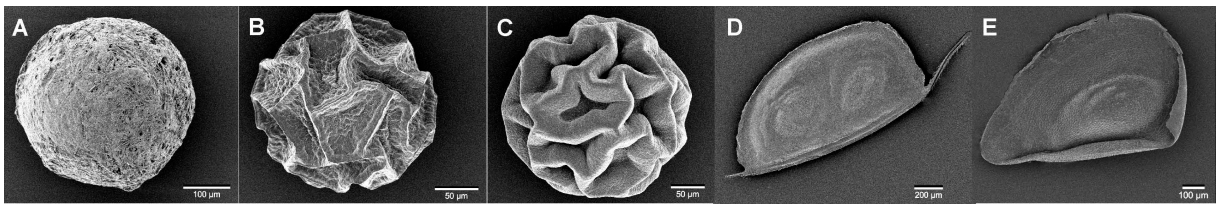


Fig. 2 Scanning electron microscope images of dormant eggs or ephippia containing dormant eggs, of different zooplankton species: A) *Triops cancriformis*, B) *Branchipodopsis wolfi*, C) *Thamnocephalus platyurus*, D) *Daphnia magna*, E) *Simocephalus* sp. (photographs by Dr. Tom Pinceel).

Dormant eggs can be dispersed to other water bodies (dispersal in space) or sink to the sediment layer (Brendonck and De Meester, 2003, Fig. 3). Dormant eggs can accumulate over the years in the sediment layer and form extensive mixed persistent dormant egg banks, analogous to plant seed banks (De Stasio, 1989; Hairston, 1996). Zooplankton dormant eggs can remain viable for hundreds of years (Hairston and Kearns, 1995; Caceres, 1998; Frisch et al., 2014). With densities in the sediment ranging between 10^3-10^6 eggs/m^2 (Hairston, 1996; Brendonck and De Meester, 2003; Vandekerkhove et al., 2005), the dormant fraction is far from negligible.

After dormant eggs are produced they enter diapause (Box 1), which can last from 2-3 months up to several years (Alekseev et al., 2007). During this period, dormant eggs are not responsive to hatching stimuli (refractory phase; Stross, 1987). Depending on, amongst others, the origin of the dormant eggs, diapause can be broken by cues, such as a cold shock, low oxygen conditions or a period of drought (Stross, 1971; Doma, 1979; Vanvlasselaer and De Meester, 2010). When diapause is broken, the eggs become quiescent (Box 1) and sensitive to hatching cues. Each growing season only a fraction of the dormant eggs hatches, while the unhatched fraction remains dormant until a new opportunity arises. Zooplankton species from permanent habitats often show less variation and a higher hatching success, than species from unpredictable temporary systems (Brendonck, 1996; Caceres and Tessier, 2003). The presence of a dormant egg bank creates a link between the active aquatic phase and the dormant benthic phase, the so called benthic-pelagic coupling (Gyllström and Hansson, 2004; Fig. 3).

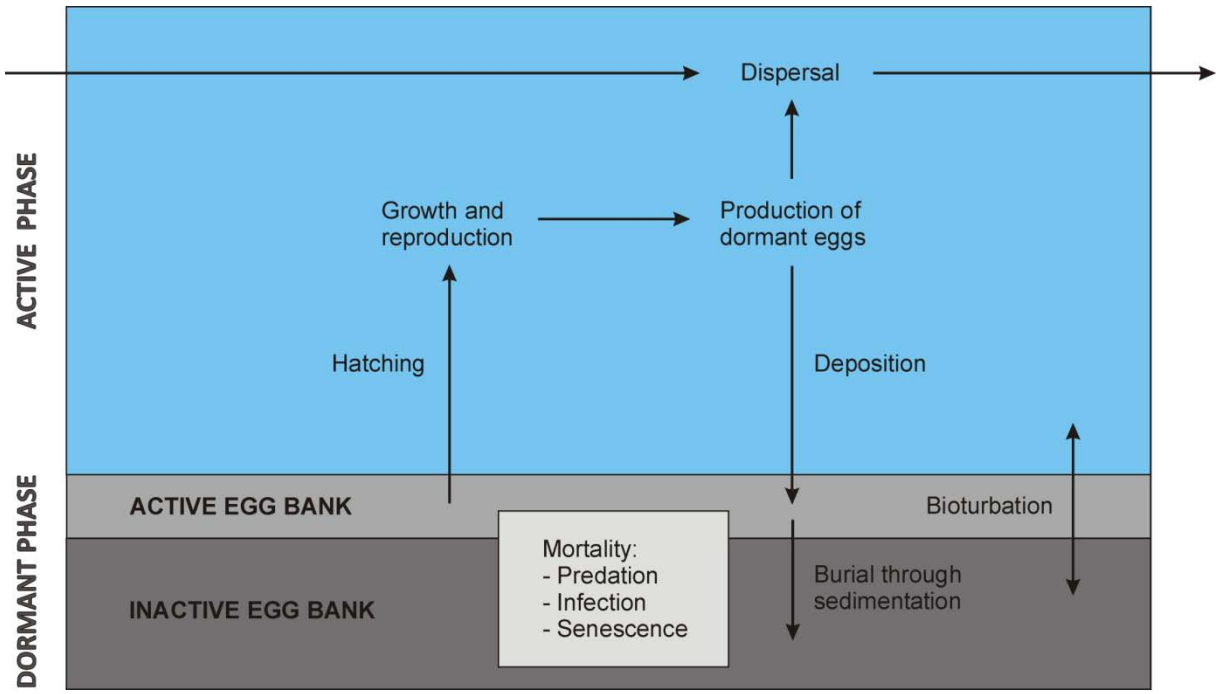


Fig. 3 Dormant egg bank dynamics and processes related to the benthic-pelagic coupling (adapted from Gyllström and Hansson, 2004).

The benthic-pelagic coupling may strongly impact ecological and evolutionary dynamics (Caceres, 1998; Gyllström and Hansson, 2004). When environmental conditions fluctuate, egg banks can function as a reservoir of species and genetic diversity through a mechanism called the "storage effect" (Chesson and Warner, 1981; Caceres, 1997), allowing coexistence of competitors that would otherwise not persist. Dormant egg banks integrate genetic variation that has accumulated over several growing seasons (Ellner and Hairston, 1994; De Meester et al., 2006), thereby creating a buffering effect in terms of population dynamics and genetic diversity (Caceres, 1997; Brendonck and De Meester, 2003).

BOX 1. Definitions related to dormant eggs (adapted from Brendonck and De Meester, 2003)

Dormancy = state of arrested development, regardless of cues needed for induction or termination, encompassing a wide spectrum of physiological states with quiescence and diapause as two extremes.

Quiescence = state of dormancy that is an immediate response to an external limiting factor. In quiescent stages metabolism and development are resumed as soon as conditions are permissible.

Diapause (refractory phase) = state of dormancy where an arrest of development is internally initiated. Diapausing organisms do not resume development, even when conditions are favorable, until diapause is broken. Conditions for breaking diapause and inducing hatching very much depend on the species and often even vary among populations of the same species.

In this thesis no distinction will be made between diapausing and quiescent eggs, unless specifically indicated, and both phenomena will be referred to as dormant or dormancy.

4. Ecotoxicology and environmental risk assessment

While toxicology is the study of adverse effects of chemicals on living organisms (Timbrell, 2001) and has traditionally focused on human health, ecotoxicology is concerned with the study of harmful effects of chemicals on ecosystems (Walker, 2014). Following the publication of the book "Silent Spring" by Rachel Carson (Carson, 1962), the term ecotoxicology was introduced by Truhaut, suggesting a scientific discipline that combined ecology and toxicology (Truhaut, 1977). During this time period concerns were raised about the toxic side effects of extensive pesticide use on natural ecosystems. This was mainly related to the use of DDT and other organochlorine pesticides that had until then been much celebrated for their efficiency and socio-economical benefits. Indeed, this class of pesticides was found to be extremely persistent, biomagnified in the food chain and was later linked to reproductive failure and population declines in birds of prey (Grasman et al., 1998). Organochlorine pesticides have since then been restricted or phased out in many countries, but are still used today in developing countries, for example in malaria control (Köhler and Triebskorn, 2013).

Currently, in Europe and the U.S., extensive legislative frameworks exist to regulate pesticides and other chemicals. To ensure safe use, pesticides are evaluated for their potential impact on the environment as well as on human health, before they can be introduced onto the market. In Europe this is governed by EC Regulation No 1107/2009 (European Parliamant, 2009). Regulatory environmental risk assessment of pesticides consists of two parts, an exposure assessment and an effect (or hazard) assessment. In the exposure assessment, concentrations in the different environmental compartments are measured or predicted, to estimate "Predicted Environmental Concentrations" (PECs). For the effect assessment, standardized tests are performed according to specific (OECD) test guidelines, following a tiered approach, and "Predicted No Effect Concentrations" (PNECs) are determined (European Commission, 2002; Walker, 2006). The comparison of PEC and PNEC values is used to assess potential risk: if PEC / PNEC < 1, there is assumed to be no risk (Walker et al., 2001).

While the ultimate protection goal in environmental risk assessment is at the ecosystem level, effects of toxicants can be studied at different levels (tiers) of biological organization, ranging from the (sub)individual level, to population, community and meta-community level (Parker et al., 1999; Walker, 2014). At the first tier of ecotoxicological effect assessment, single-species aquatic and terrestrial ecotoxicity tests are performed (Bradbury et al., 2004; EFSA, 2013). For the aquatic component, typically organisms of three trophic levels are considered: algae and/or macrophytes (primary producers), invertebrates (primary consumers) and fish (secondary consumers) (De Jong et al., 2008; Walker, 2014). In these screening tests, biochemical and physiological effects may occur on a relatively short time scale and can often be directly linked to chemical exposure. However their ecological relevance might be limited. If this first screening indicates potential concerns, registrants are required to carry out further higher tier testing and demonstrate there is no unacceptable impact on non-target organisms under realistic field conditions (EC, 2009). Studies of toxicant effects on the population or community level have a greater ecological relevance, but the causality of effects is often difficult to assess (EPiF, 2005; Köhler and Triebskorn, 2013; Box 2).

BOX 2. Overview of strengths and limitations of lower and higher tier ecotoxicity tests

Single-species laboratory experiments	**Outdoor mesocosm / field studies**
- Single species	+ Represent aquatic communities
- Model organisms	+ Include species interactions
- Laboratory conditions	+ (Semi-)natural conditions
- Short time-span (hours-weeks)	+ Longer time-span (months-years)
+ Direct link between stressor and effect	- Causality of effect chemical stressor ?
+ Repeatability between experiments and laboratories	- Variability (time, replicates)
	- Costly

Artificial model ecosystems (e.g. micro- or mesocosms) are often used as surrogates for actual field testing or surveys (Campbell et al., 1999; De Jong et al., 2008). Compared to single species ecotoxicity tests, mesocosm studies show more ecological realism and allow to consider species interactions, indirect effects and the potential for communities to recover from, for example, pesticide exposure (Boxall et al., 2002; Solomon and Sibley, 2002; Box 2). In general, tests across the different levels of biological organisation are deemed necessary to assess potential impacts of toxicants and provide sufficient data for environmental risk assessment (Clements, 2000). However, ecotoxicological effect assessments for regulatory purposes are mainly based on single species tests, making extrapolation to potential effects at the population or community level questionable. Over the past decades there has been a growing interest in the development of more sophisticated and ecologically relevant methods for ecotoxicity testing and environmental risk assessment (e.g. Chapman, 2002; Breitholtz et al., 2006; Relyea and Hoverman, 2006; Schmitt-Jansen et al., 2008). Indicated as important issues were, amongst others, increasing long-term testing of toxicants under environmentally relevant exposure conditions, focusing on multiple ecologically relevant species (and contaminants), and testing the full or most sensitive part of their life-cycle.

5. Impact of pollutants on dormant egg bank dynamics

Anthropogenic stressors could impact both the active and dormant phase of zooplankton populations and communities, through the following scenario´s (adapted from Angeler and Garcia, 2004; Fig. 4): 1) impact on development and hatching of dormant eggs; 2) effects on hatchling survival and performance in the aquatic phase; 3) impact on dormant stages before activation (in diapause), causing egg mortality or irreversible disruption of the dormancy-break system; 4) effects during the sexual reproductive phase, affecting dormant egg production. While aquatic invertebrates are routinely tested in ecotoxicological studies (especially the model organism *Daphnia magna*), most of these studies focus on a small part of their life cycle: asexual reproduction of clonal lineages (scenario 2). Studies investigating effects of pollutants on dormant life stages and the sexual reproductive phase are vastly underrepresented (Moest et al., 2015; scenario 1, 3 and 4).

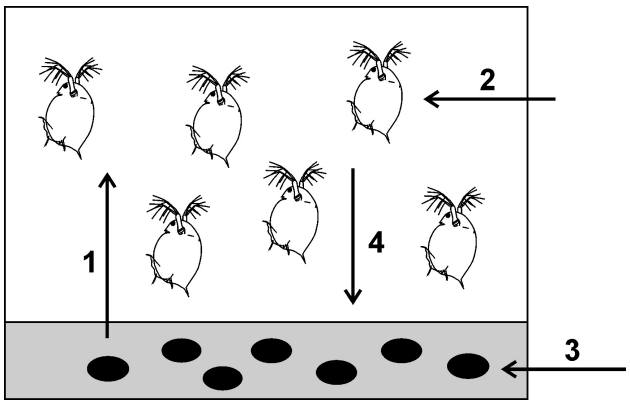


Fig. 4 Potential scenarios through which toxicants can impact the active and dormant phase of zooplankton populations and communities:
1) impact on hatching process;
2) effects on hatchling survival and performance;
3) impact on dormant stages before activation;
4) effects during sexual reproductive phase.
Also combinations of one or more of the above mentioned scenarios are possible.

Several studies have assessed the effects of various types of contaminants on the viability and hatching process of zooplankton dormant eggs (scenarios 1 and 3). The biocides sodium hypochlorite and menadione were tested to determine their potential for use in treating ship ballast tanks, in order to avoid introduction of exotic/invasive species through their dormant stages. Both biocides affected hatching success of *D. mendotae* dormant eggs, but effect levels were higher compared to other *D. mendotae* life stages (Raikow et al., 2006; Raikow et al., 2007). To evaluate potential effects of fire retardant treatment on wetlands, Angeler et al. (2005) exposed wetland sediments to the fire retardant Fire Trol 943 and found a significant negative effect on emergence of the focal species *D. curvirostris*. At a zooplankton community level, they observed a decrease in taxon richness and hatchling abundances after fire retardant exposure (Angeler et al., 2006). Henri et al. (2014) also observed negative effects of toxicant exposure on a zooplankton community level; hatching of dormant eggs was severely impaired after exposure to mining effluent, mainly containing heavy metals. Marcial et al. (2005) tested several pesticides (diazinon, fenitrothion, methoprene and isoprothiolane) and concluded they affected hatching of the rotifer *Brachionus plicatilis* dormant eggs, even at lower concentrations than affecting growth and reproduction. Copepod dormant eggs (*Acartia pacifica)* were also shown to be much more sensitive to heavy metal (copper, lead and cadmium) exposure than benthic adults (Jiang et al., 2007). Several tests have been performed on *Artemia* cysts, but the results are inconclusive. Varó et al. (2006) and Sarabia et al. (2003, 2008) reported no adverse effects of metals (zinc and mercury) and pesticide (chlorpyrifos) exposure on hatching of *Artemia* cysts. Bagshaw et al. (1986) and Rafiee et al. (1986), on the contrary, reported that hatching of dormant eggs was more sensitive to heavy metal (cadmium and zinc) exposure than hatched individuals. Moest et al. (2015) studied hatching success and hatchling survival of *D. longispina* ephippia, after exposure to a mixture of organic contaminants. This was the first study to report an increase in hatching success after exposure to toxicants, combined with a decrease in hatchling survival.

A number of studies have shown that pesticides, mainly insect growth regulators, are able to affect sexual reproduction in aquatic inverbetrates (Olmstead and LeBlanc, 2000, 2003; Tatarazako, 2003; Wang et al., 2005; scenario 4). These chemicals induced the production of male offspring in *Daphnia* clones, that produce female offspring under control conditions (Oda et al., 2005). In addition, methoprene, also an insect growth regulator, was able to significantly reduce dormant egg production in *D. magna* (Olmstead and LeBlanc, 2001a). This was also observed for the surfactant nonylphenol (Shurin and Dodson, 1997) and the biocide tributyltinoxide (Saika et al., 2006). Marcial and Hagiwara (2007) exposed the rotifer *B. plicatilis* to the pesticide diazinon during dormant egg production and they observed that hatching success of eggs exposed during or shortly after production was severely affected.

6. Research questions and general methodology

As introduced in the previous sections, the ecological realism of standardized testing for ecotoxicological effect assessment is relatively low. In addition, despite its ecological importance, our understanding of the effects of toxicant exposure on zooplankton dormant egg bank dynamics is limited. With this PhD research we aim to provide a better understanding of the effects of toxicant exposure on dormant egg bank dynamics, by studying different endpoints related to the dormant component in zooplankton populations and communities.

Specifically, we posed the following research questions:

1) Which part of the life-cycle in the model organism *D. magna* is most sensitive to toxicant exposure? Are standard first-tier screening studies using *D. magna* sufficiently conservative?
2) What new information regarding the sensitivity and recovery potential of aquatic communities can be obtained from including effects on dormant egg bank dynamics in higher tier ecotoxicological studies?

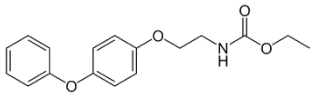
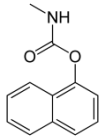
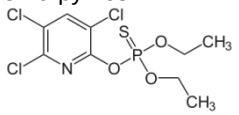
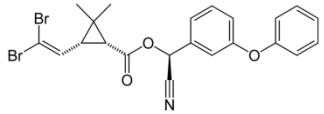
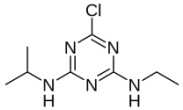
To investigate these issues we used zooplankton communities from permanent aquatic systems in Belgium, with as focal species the model organism *D. magna*. We tested the effects of a number of different model pesticides with a different mode of action, in both laboratory experiments and a mesocosm study.

7. Model pesticides

Pesticides are chemicals that are “used with the intention of causing damage to pest species and other organisms that threaten the health or well-being of humans” (Walker, 2014). For the experiments in this thesis, we have used five model pesticides with a different mode of action: fenoxycarb, carbaryl, chlorpyrifos, deltamethrin and atrazine (Table 1).

Fenoxycarb is an insect growth regulating insecticide (IGR), which specifically attacks the endocrine system in insects (EFSA, 2010). Fenoxycarb mimics juvenile hormones, that are naturally present in insects and involved in growth and development. Under normal conditions, a drop in the level of juvenile hormones in insects causes insects to molt or metamorphose into a next life-stage. When exposed to IGRs, juvenile hormones continue to circulate, signaling the organism to stay in its current life stage (Brown, 2006). In addition to mimicking juvenile hormones present in target insects (Lepidoptera and *Tortricidae* in fruit crops), several studies have demonstrated that IGRs are also able to mimic methyl farnesoate. This is an important terpenoid hormone in crustaceans involved in the regulation of embryonic development, growth and reproduction (reviewed in LeBlanc, 2007). Exposure to juvenile hormone mimicking substances has been shown to impact reproduction in zooplankton species, at very low concentrations (Olmstead and LeBlanc, 2001b; Abe et al., 2015).

Table 1. Overview of the model pesticides used in this thesis, their structure, chemical class, mode of action, half-life in water-sediment systems and octanol-water partitioning coefficient (log K_{ow}) (source: Pesticide Properties Database). In addition effect levels in the model organism *Daphnia magna* are summarized, as well as European environmental quality standards and measured concentrations in surface waters.

Pesticide	Chemical class	Mode of action	DT_{50} water-sed (days)	log K_{ow}	Effect levels in *Daphnia*		Env. quality standards		Measured concentrations In surface waters	
					48h EC50 (µg/L)	21d NOEC (µg/L)	AA-EQS (µg/L)	MAC-EQS (µg/L)	Annual average levels (ng/L) [1]	Peak levels (µg/L)
Fenoxycarb 	Insect growth regulator	Juvenile hormone mimicking	15	4.1	500 [2]	0.002-3.2 [2]	0.0003	0.026	7.1 (n = 1899)	0.5 [8]
Carbaryl 	Carbamate insecticide	Acetylcholine esterase inhibitor	6 (pH sensitive)	2.4	6-17 [3,4]	n.d. [4]	0.23	–	12.0 (n= 1569)	1737 [9] 5 - 4800 [10]
Chlorpyrifos 	Organophosphate insecticide	Acetylcholine esterase inhibitor	37	4.7	0.1-0.7 [5,6]	4.6 [5,6]	0.03	0.1	4.6 (n = 2378)	0.4 [11] 1.2 - 486 [12]
Deltamethrin 	Pyrethroid insecticide	Sodium channel modulator	65	4.6	0.1-0.6 [5,7]	0.004 [5,7]	0.0000031	0.00031	15.7 (n = 2595)	1.4 [13]
Atrazine 	Triazine herbicide	Blocking photosynthesis	80	2.7	3550 [5,6]	250 [5,6]	0.6*	2	6.1 (n = 2176)	0.6 [14] 2.3 - 5.9 [15] 16 - 47 [16]

[1] Pesticide Atlas (the Netherlands, 2013); [2] EFSA (2010); [3] Coors et al. (2009); [4] EFSA (2006); [5] PPDB; [6] Palma et al. (2008); [7] EC (2003); [8] Süß et al. (2006); [9] Walters et al. (2003); [10] Bridges et al. (1999); [11] US EPA (1999); [12] Palma et al. (2008); [13] Dabrowski et al. (2002); [14] Cerejeira et al. (2003); [15] Konstantinou et al. (2006); [16] Maloschik et al. (2007).

Carbamate insecticides, such as carbaryl, and organophosphate insecticides, like chlorpyrifos, are both readily biodegradable and have the same mode of action. They act as cholinesterase inhibitors, causing overstimulation of the nervous system (Brown, 2006). By binding to the enzyme acetylcholine esterase, that is normally responsible for breaking down acetylcholine in the nerve synapses, acetylcholine builds up and signaling becomes continuous, preventing muscles to relax and respond to subsequent synaptic stimuli (Walker, 2014). Binding of carbamate insecticides is readily reversible, whereas binding of organophosphate insecticides is irreversible. Both pesticides are highly acutely toxic to a wide range of organisms, especially birds and aquatic invertebrates (EFSA, 2006).

Deltamethrin is a pyrethroid insecticide, that can affect the nervous system by acting on sodium channels of nerve membranes, resulting in continuous nerve impulse transmissions and tremors (Brown, 2006). Deltamethrin is widely used on green areas, a variety of crops and vegetables, against e.g. mites, ants and beetles (EC, 2003). In the environment deltamethrin binds to soil and sediments, but is highly toxic to fish and aquatic invertebrates while in the water phase (Toumi et al., 2013) and is reported to cause algal blooms in aquatic systems (Bhanu et al., 2011). There has been some concern and controversy about possible side effects of deltamethrin exposure on bees and other pollinators (Dai et al., 2010; Walker, 2014).

Atrazine is the only herbicide we used as model pesticide. It inhibits photosynthesis in plants and is mainly used for the control of grassy and broadleaf weeds (especially corn). It is one of the most widely applied herbicides in the U.S. and commonly detected in surface waters (EPA, 2013). Atrazine has a low acute toxicity, but there are some reports on potential endocrine effects in invertebrates (Olmstead and LeBlanc, 2003; Stoeckel, 2008; Palma et al., 2009).

8. Study populations and communities

As starting material for the experiments performed in this thesis, we used dormant eggs from natural populations and a laboratory population. For the natural populations, we selected five permanent shallow lakes, all situated in the region around Leuven, Belgium: Langerodevijver (LRV), Oud-Heverlee Noord (OHN), Oud-Heverlee Zuid (OHZ), Oud-Heverlee P (OHP) and Zoete Waters 4 (ZW4). Since land use intensity around water bodies (especially distance to crops) was proven to be an important parameter influencing the tolerance of *D. magna* populations to toxicant exposure, we selected only lakes that were not surrounded by crops for at least 200 m (Google Earth, photographs 2009-2010). In addition, all of these lakes are known to contain a high density of cladoceran dormant eggs, including *D. magna* ephippia (Louette et al., 2007; Coors et al., 2009; Tom de Bie, pers. com.). We sampled the active egg bank (Caceres, 1998) of these lakes in 2011. LRV was sampled again in 2012 and 2013. Sediment was always collected in the period between December to February, when dormant eggs in the sediment were no longer in the refractory (unresponsive) phase as they had already received a natural cold shock, but before the main zooplankton hatching peak, that in these systems normally takes place in early spring (Vandekerkhove et al., 2005). Sediment with dormant eggs was stored at 4°C in the dark, until further use in the experiments.

In addition to dormant eggs from the natural populations, we also used *D. magna* dormant eggs from a standardized laboratory culture (MicroBioTests Inc, Mariakerke, Belgium). These dormant eggs are internationally used as starting material to obtain neonates for use in standard ecotoxicity assays. Hatched neonates of these laboratory cultures have a sensitivity to reference toxicants that is similar to parthenogenetic offspring from standardized laboratory clones (Persoone et al., 2009).

9. Model organisms

In our experiments we studied effects at the population level, using *D. magna* as a model species. In addition, we also studied effects at the zooplankton community level (Fig. 5). *Daphnia* is a well-established model organism in ecological, eco-genomical and evolutionary research (Lampert and Kinne, 2011; Miner et al., 2012). They are also frequently used as model organisms in ecotoxicological research, as representative for aquatic invertebrates, based on their relative sensitivity to toxicant exposure, fast generation time and easy laboratory culturing (Lampert and Kinne, 2011; Altshuler et al., 2011).

Fig. 5 A selection of zooplankton species present in our study communities: A) *Ceriodaphnia quadrangula,* B) *Chydorus sphaericus,* C) *Scapholeberis mucronata* and D) *Alona rectangula (*photographs by Dr. Ralf Wagner).

Species of the genus *Daphnia* (Branchiopoda, Cladocera, Daphniidae) are planktonic crustaceans, that can be found in a wide variety of water bodies, ranging from temporary rock pools to large lakes (Ebert, 2005). Especially in temperate regions, *Daphnia* are often one of the most significant components of zooplankton communities. They play a key role in many aquatic systems, both as the preferred food source for macro-invertebrates and planktivorous fish (secondary consumers), and as the main grazers of phytoplankton (primary producers) (Miner et al., 2012). Like many other cladocerans, most *Daphnia* species reproduce by cyclical parthenogenesis, which is a mixed reproductive strategy, combining both sexual and asexual reproduction (Bulmer, 1982; De Meester et al., 2004).

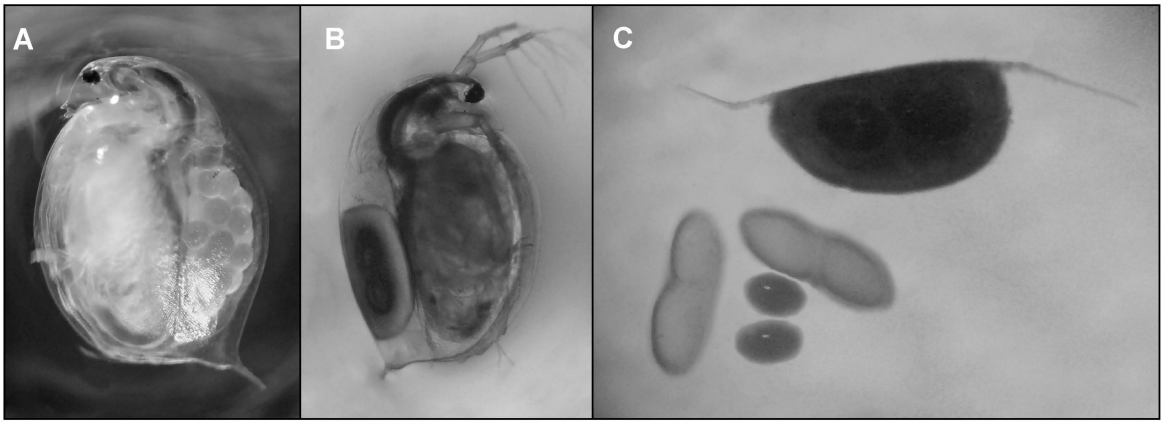


Fig. 6 A) *D. magna* parthenogenetic female; B) *D. magna* ephippial female; C) Opened *D. magna* ephippium with two dormant eggs and their inner envelope (two halves) which normally surrounds the dormant eggs.

During the growth season, when environmental conditions are favourable, *Daphnia* reproduce clonally (Fig. 6A). After every molt females produce a clutch of parthenogenetic eggs, that develop directly into genetically identical females. When environmental conditions deteriorate, they can switch to sexual reproduction (Innes and Singleton, 2000; Decaestecker et al., 2009). The production of males is induced, which in turn fertilize sexual females, leading to the formation of dormant eggs (Fig. 6B). These dormant eggs are encapsulated in an protective structure, called ephippium (Fig. 6C), consisting of a chitinuous membrane that is formed around the brood pouch (Schulz, 1977). With the subsequent moult, the old carapax including the ephippium is shed (Zaffagnini, 1987). Since these dormant eggs do not hatch immediately, in *Daphnia* sexual reproduction is linked to dormancy (Alekseev et al., 2007; Miner et al., 2012; Box 1). Once quiescent, hatching can in general be induced by exposure to conditions that mimic spring: increase in temperature, long-day photoperiod and changing of medium (Alekseev et al., 2007; Vanvlasselaer and De Meester, 2010).

Parthenogenetic eggs are surrounded by two membranes: an outer membrane (0.35 µm thick) and a thin inner membrane. Dormant eggs have thick multi-layered membranes: an outer membrane (0.70 µm), a middle membrane (1.40 µm) and a thin inner membrane (0.10 µm) (Seidman and Larsen, 1979; Zaffagnini, 1987). In addition, dormant eggs are encapsulated in an ephippium (Schultz, 1977; Ebert, 2005:). Both the ephippium and the thick membrane structure, protect dormant eggs from mechanical damage and digestive enzymes of organisms, like fish and birds (Mellors, 1975; Radzikowski, 2013). An ephippium can contain zero, one or two dormant eggs.

10. Outline of the thesis

The aim of this PhD research is to provide a better understanding of the effects of pesticide exposure on dormant egg bank dynamics, by studying different endpoints related to the dormant component in zooplankton populations and communities. The thesis consists of two main parts (schematic overview in Fig. 7). The first part focuses on population level effects, by investigating the impact of pesticide exposure on different endpoints related to dormant life stages of the cyclic parthenogenetic model organism *D. magna,* in a laboratory setting. The second part focuses on effects of pesticide exposure at the community level, looking at effects both on the active and dormant component of zooplankton communities in semi-natural aquatic model ecosystems (i.e. mesocosms).

To get more insight into the acute and chronic effects of pesticide exposure on *D. magna* dormant eggs, we have conducted a series of laboratory experiments (**chapters 1 - 4**). Experiments presented in **chapters 1 - 3** focus on pesticide exposure of dormant eggs during, or just prior to, the hatching process. In **chapter 1** we screened five model pesticides with a different mode of action for their effects on development and hatching of *D. magna* dormant eggs. We compared these effects with effect levels in parthenogenetic eggs. In **chapter 2** we used two of the previously tested model pesticides (carbaryl and fenoxycarb), to not only test for direct effects of pesticide exposure on development and hatching of the dormant eggs, but also to test for effects on survival and life-history characteristics of hatched individuals. In **chapter 3** we wanted to reveal the most sensitive embryonic developmental stage for pesticide exposure (fenoxycarb). In addition, we assessed the potential for pesticide bioaccumulation into the dormant eggs, during different stages of their development. We also compared effects on decapsulated dormant eggs versus dormant eggs encapsulated in their ephippium, to better understand the protective value of the ephippial casing against chemical exposure.

In **chapter 4** we tested for effects of pesticide exposure during the sexual reproductive phase. We exposed a mix of male and female *D. magna* to a pesticide (fenoxycarb), while simultaneously inducing sexual reproduction. This allowed us to test for impact on sex ratio of parthenogenetic offspring and effects on dormant egg production. We subsequently used the produced dormant eggs in a hatching and a life table experiment to assess whether pesticide exposure during dormant egg production had effects on embryonic development, hatching, and life history parameters of hatched individuals.

To better understand the environmental relevance of our findings in the laboratory experiments, we conducted a two year outdoor mesocosm experiment of which the results are presented in the second part of this thesis (**chapter 5**). In this experiment, the impact of repeated pesticide exposure (carbaryl) on both the active and dormant component of zooplankton communities was studied. To test specifically for effects of pesticide exposure on newly produced dormant eggs (quantity and quality) as well as on dormant eggs already present in the sediment (to assess the buffering capacity of the egg bank), both a pesticide and a dormant egg bank treatment were included in our experiment.

Finally, we integrated the conclusions of all chapters in a **general discussion** section in which we discuss the importance of our findings from both an ecological and ecotoxicological perspective.

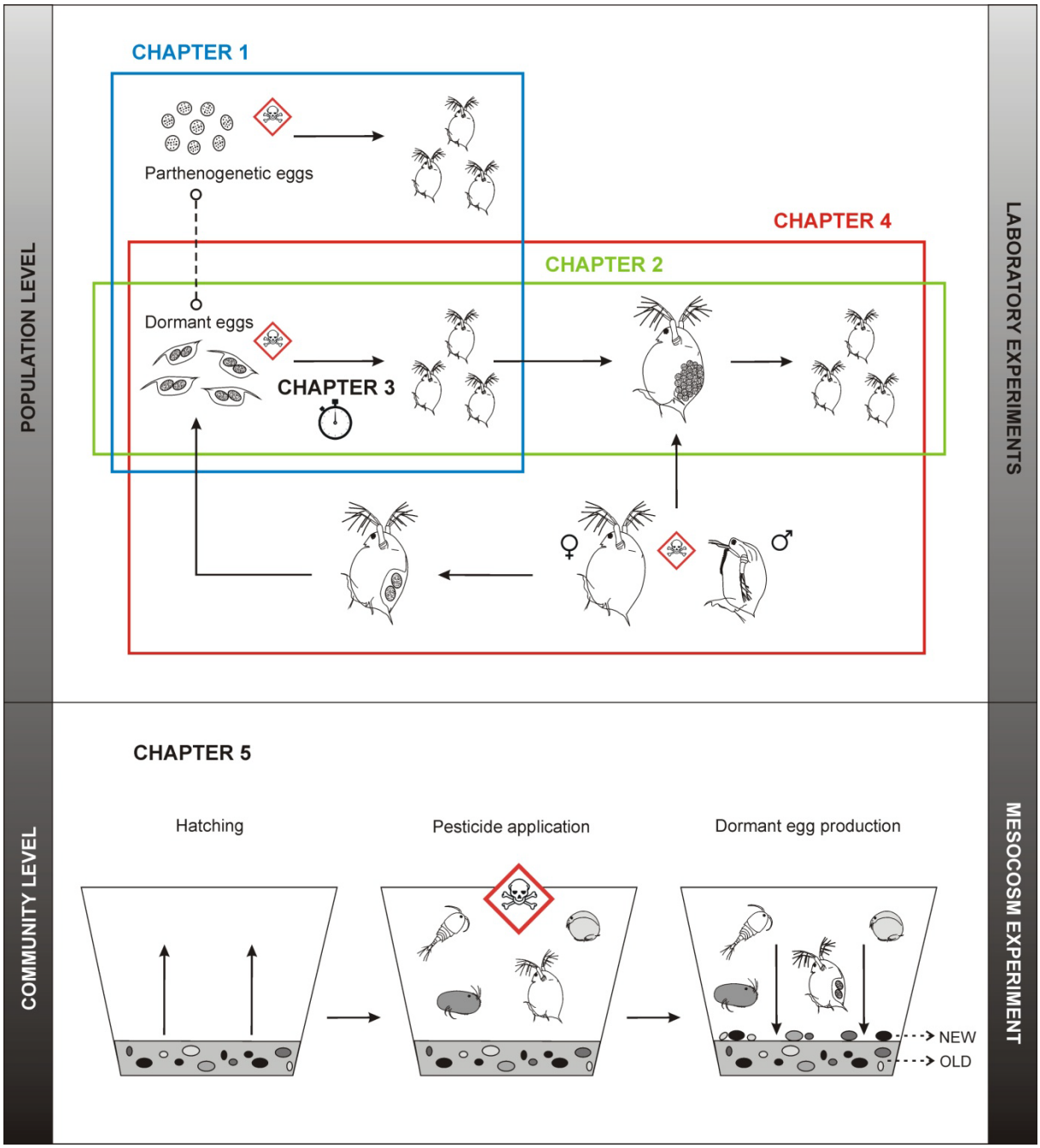


Fig. 7 Schematic overview and general outline of the two major parts of this thesis: effects of pesticide exposure on dormant life stages were studied at the zooplankton (*Daphnia magna*) population (**chapters 1-4**) and community level (**chapter 5**).

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PART I: POPULATION LEVEL

CHAPTER 1

Susceptibility of *Daphnia magna* eggs to pesticides: a comparison between reproductive strategies

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Unpublished manuscript

Abstract

In the framework of environmental risk assessment, chemical substances are frequently tested on *Daphnia* in acute and chronic ecotoxicological tests according to internationally accepted guidelines, focusing on neonate mortality and reproductive endpoints of clonal lineages. In addition to these standard tests, short in vitro assays have been used to assess potential teratogenic effects of chemicals using parthenogenetic eggs. However, *Daphnia* reproduce by cyclical parthenogenesis, alternating asexual (clonal) and sexual reproduction. During the sexual reproductive phase, dormant eggs are produced, that can survive harsh environmental conditions and remain viable for decades to centuries. Even though they are essential for the long-term persistence of *Daphnia* populations in natural aquatic ecosystems, not much is known about the effects of chemical exposure on these dormant eggs. To compare the sensitivity of parthenogenetic and dormant eggs to pesticide exposure, we screened five pesticides with a different mode of action for their impact on hatching and development of both parthenogenetic and dormant eggs of *D. magna* from the same population. Our results show that all model pesticides are able to affect the hatching process in both egg types, mainly by inducing malformations in developing embryos. Fenoxycarb was the only model pesticide that also negatively affected hatching in dormant eggs. The severity of the deformations and effect levels differed between pesticides and egg types. In general, more severe effects (deformations of carapax and second antennae) were observed at the highest tested exposure concentrations for all pesticides, except atrazine, and occurred at 10-fold lower concentrations in parthenogenetic eggs compared to dormant eggs. When comparing our results with effect levels measured in traditional screening tests using *D. magna* neonates, it was revealed that the most sensitive life stage was pesticide dependent.

Introduction

In the framework of environmental risk assessment, chemicals are screened for their potential toxic effects on aquatic ecosystems. In general, organisms of three trophic levels are tested: algae and/or macrophytes (primary producers), invertebrates (primary consumers) and fish (secondary consumers) (Walker, 2014). Species of the genus *Daphnia* (Branchiopoda, Cladocera) are frequently used as model organism representing aquatic invertebrates, based on their relative sensitivity to toxicant exposure, fast generation time and easy laboratory culturing (Lampert and Kinne, 2011). Traditionally, chemical substances are tested on *Daphnia* in an acute 48-hour test based on neonate mortality/immobility (OECD Guideline no. 202; OECD, 2004) and in a chronic 21-day test measuring reproductive endpoints (OECD Guideline no 211; OECD, 2012). In addition to these standard assays, several studies have assessed potential teratogenic (developmental) effects of chemicals using short *in vitro* assays with *Daphnia* embryos (Ohta et al., 1998; Sobral, 2001; Kast-Hutcheson et al., 2001; Mu and LeBlanc, 2002, 2004; Palma et al, 2009a, 2009b, 2011). These assays use parthenogenetic eggs, which are genetically identical and easy to collect in large quantities (Ohta et al., 1998; Kast-Hutcheson et al., 2001). Combined with a fast developmental time (about three days) and generally high hatching success, parthenogenetic eggs are very suitable for use in ecotoxicological screening tests (Palma and Barbosa, 2011). Because a number of pesticides were found to cause a dose-related increase in developmental abnormalities after embryonic exposure (e.g. Kast-Hutcheson et al., 2001; Mu and Leblanc, 2004; Hassold and Backhaus, 2009; Palma et al., 2009a, 2009b) at effect levels comparable to or lower than those found in the classic *Daphnia* assays, this early life-stage test is proposed by some researchers as a fast, cost-effective alternative to the classic 21-day *Daphnia* reproduction assay (Palma et al., 2009a; Sobral, 2001; Abe et al., 2015).

Except for some obligately parthenogenetic strains, most *Daphnia* species, including the most commonly used, *D. magna*, do not reproduce solely by parthenogenetic reproduction (Decaestecker et al., 2009). *Daphnia* switch from asexual reproduction to the sexual production of dormant life stages when conditions deteriorate (Ebert, 2005; Walsh, 2013). These encapsulated dormant eggs (ephippia) are resistant to freezing, drought and predation (Mellors, 1975; Radzikowski, 2013), enabling populations to persist even in harsh and unpredictable environments (Gyllström and Hansson, 2004; Brendonck and De Meester, 2003). Despite its ecological importance, the sexual part of the reproduction cycle in *Daphnia* is generally not taken into account in ecotoxicological studies (Simpson et al., 2014). To date not much is known about the sensitivity of dormant eggs to chemical exposure (Brendonck and De Meester, 2003). The few studies that have been done so far indicate that both decapsulated and encapsulated dormant eggs of *Daphnia* can be affected by toxicant exposure (Angeler et al., 2005; Raikow et al., 2006, 2007; Navis et al., 2015; Chapter 3). For rotifers, hatching of sexual dormant eggs was found to be even more sensitive to pesticide exposure than asexual eggs (Marcial et al., 2005; Marcial and Hagiwara, 2007).

With this study, we aim to improve our understanding of the effects of chemical exposure on *D. magna* eggs during the hatching process, using a simple screening method. Therefore, we selected five model pesticides with a different mode of action, which are known to affect development of *D. magna* parthenogenetic eggs (Mu and Leblanc, 2004; Palma and Barbosa, 2011; Palma et al., 2009a, 2009b; Toumi et al., 2013). We compared the impact of these pesticides on development and hatching of both parthenogenetic and decapsulated dormant eggs of *D. magna* from the same population. Dormant eggs are surrounded by an additional membrane (Zaffagnini, 1987) and are able to survive extreme physical conditions (Radzikowski, 2013). We therefore expected decapsulated dormant eggs to be less sensitive to pesticide exposure than parthenogenetic eggs.

Material & Methods

Collecting parthenogenetic and dormant Daphnia magna *eggs*

As starting material for the experiments we used ephippia isolated from the sediment of Langerodevijver, a shallow lake located in rather pristine environment (natural area "Doode Bemde", Leuven, Belgium). Sediment of this lake contains a high density of *D. magna* ephippia, with a hatching success of 80-90% under optimal hatching conditions and after decapsulation (Navis et al., 2013, 2015; Chapter 2, 3). The top 5-10 centimeters of the dormant egg bank (active egg bank: Caceres, 1998) was sampled in the winter period of 2012-2013. Pooled sediment samples were sieved (1 mm and 250 µm sieves) and stored for a minimum of one year at 4°C in the dark, before ephippia were manually isolated. All manipulations were performed in a room with only red light (700 nm), to prevent unwanted activation of the dormant eggs by light exposure. The storage period ensured that diapause was terminated, so that the eggs became quiescent and hatching could be induced under favorable conditions (Stross, 1971; Vandekerkhove et al., 2005). Isolated ephippia containing dormant eggs were kept under storage conditions until the start of the experiment. Shortly before the start of the experiment, they were mechanically decapsulated with metal tweezers. Decapsulated dormant eggs were used for the experiments (as opposed to encapsulated eggs) in order to track the development of the embryos, to maximize exposure, and to have an exposure regime comparable to that of the parthenogenetic eggs.

To produce parthenogenetic eggs, 100 ephippia were first hatched in the laboratory and hatchlings were cultured for approximately one year in the laboratory (temperature 20±2°C, photo regime of 16h light : 8h dark, feeding on *Scenedesmus obliquus*). Two months before the start of the experiment, 15 individuals per clone (eight clones in total) were transferred to 1 L beakers containing ADaM-medium (Klüttgen et al., 1994), fed daily with *Scenedesmus obliquus* ($1*10^5$ cells/mL) and medium was renewed every two days. Adult females from these eight clones were used to collect parthenogenetic eggs of their second and third brood. Females were screened daily and when containing eggs in the first developmental stage (Kast-Hutcheson et al., 2001), eggs were flushed out of the brood pouch using dissection needles and a pipette.

Model pesticides

For the experiment, five model pesticides with a different mode of action were selected: 1) fenoxycarb, a juvenile hormone mimicking insecticide (CAS no. 72490-01-8); 2) carbaryl, an acetylcholine esterase inhibitor (reversible binding), carbamate insecticide (CAS no. 63-25-2); 3) chlorpyrifos, an organophosphate insecticide, irreversibly inhibiting acetylcholine esterase (CAS no. 2921-88-2); 4) atrazine, a triazine herbicide, blocking photosynthesis (CAS no. 1912-24-9); and 5) deltamethrin, a sodium channel modulator, pyrethroid insecticide (CAS no. 52918-63-5). All chemicals used were analytical standards (minimum purity of 99.8%), purchased from Sigma-Aldrich (Germany). Except for carbaryl, all selected pesticides were previously tested with *D. magna* parthenogenetic eggs and reported to have effects on embryonic development (Mu and Leblanc, 2004; Palma et al., 2009a; Palma and Barbosa, 2011; Toumi et al., 2013). Atrazine was the only herbicide used as model pesticide and was selected based on reports of potential endocrine effects in different invertebrate species (Olmstead and LeBlanc, 2003; Stoeckel, 2008; Palma et al., 2009), indicating atrazine not only affects targeted plants, but it can have a broader ecological impact on non-target organism groups.Test concentrations used in our experiment were based on effect levels for hatching and embryonic development as reported in these studies and on the results of pilot experiments (S. Navis, unpublished data). Although no information was available for its effect on parthenogenetic eggs, we selected carbaryl because previous research indicated chronic effects on hatchling survival after exposure of dormant eggs (Navis et al., 2013; Chapter 2). Carbaryl test concentrations were based on the sensitivity of *D. magna* neonates in standard acute toxicity tests (Coors et al., 2009; EFSA, 2006) and earlier hatching experiments with dormant eggs (Navis et al., 2013; Chapter 2). All test substances were dissolved in absolute ethanol (purity min. 99.8%, VWR International, France). The concentration of ethanol was the same in all treatment solutions and in the solvent control (0.05% ethanol).

Hatching experiment

In a hatching experiment development and hatching of *D. magna* dormant and parthenogenetic eggs were monitored upon exposure to the five pesticides. For parthenogenetic eggs, we tested for each pesticide four concentrations and two controls (blank and solvent): in total 5 x 4 = 20 treatments + 2 controls. Carbaryl, fenoxycarb and atrazine were tested at 0.1, 1, 10 and 100 µg/L. Chlorpyrifos and deltamethrin were tested at 0.01, 0.1, 1 and 10 µg/L. All test concentrations mentioned in this test are nominal exposure concentrations. In each of the 22 treatments, 48 parthenogenetic eggs were exposed, with six eggs per clone per treatment. We specifically choose 48 eggs to be able to test a number of different clones (8 clones), representing the natural variation present within the study population. In addition, we aimed to have a sufficient number of eggs per treatment allowing detection of deviations from hatching success and development in the control treatments. Eggs were placed individually into the wells of a 48-wells plate (polystyrene, non-coated, sterile plates, Greiner Bio-One GmbH) and exposed to the respective pesticide concentration at 20±2°C and a photoperiod of 16h light : 8h dark.

Because we expected dormant eggs to be less sensitive, the concentration gradient was expanded with one additional test concentration, another factor of 10 higher (in total 5 x 5 = 25 treatments + blank and solvent control). In each treatment 48 dormant eggs were tested, which is equal to the number of parthenogenetic eggs tested per treatment. Eggs were placed individually in the wells of a 24-wells plate and exposed to the respective pesticide concentration. Treatments were randomized over plates in such a way that each treatment was allocated to four randomly assigned half multiwell plates. Hatching was initiated by light activation (47.2 µmoles/m^2/s) at 20±2°C and a photoperiod of 16h light: 8h dark.

Hatching characteristics (hatching success and timing of hatching) and developmental malformations were subsequently monitored in all plates for 10 days. A hatchling with only deformations of the tail spine was considered to have minor deformations, since this type of deformations is generally reversible when hatchlings are reared under optimal conditions (Mu and Leblanc, 2004; S. Navis, personal observation; Fig. 1D). Hatchlings and embryos with additional deformations of the carapax and/or second antennae were considered severely deformed (Fig. 1B+E).

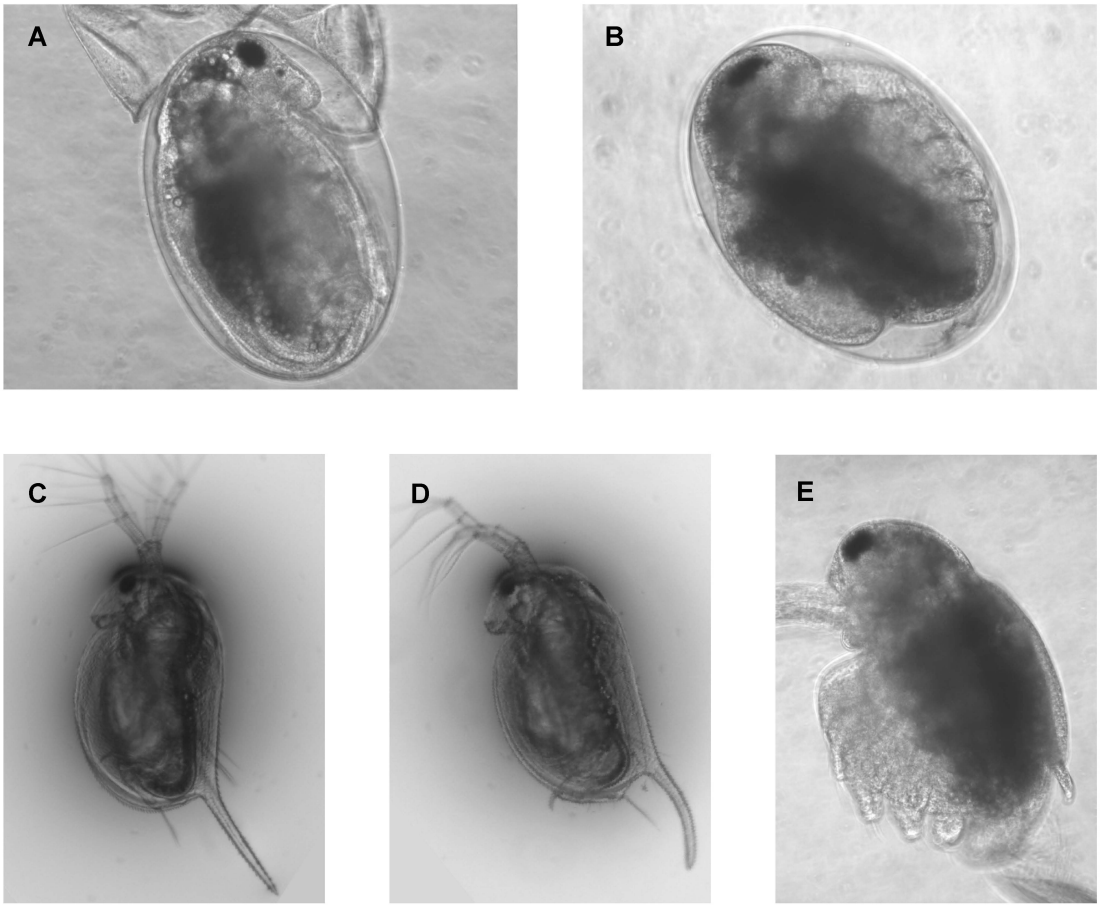


Fig. 1 Photographs illustrating pesticide induced deformations in *D. magna* dormant eggs and hatchlings. On the top row *D. magna* embryos in the final developmental stage before hatching, A) a dormant egg from control treatment, and B) from a pesticide treatment (deformations of the carapax, second antennae and compound eye). On the bottom row *D. magna* hatchlings of dormant eggs with C) no deformations (control treatement), D) minor deformations (tail spine), and E) severe deformations (carapax, tail spine, antennae, compound eye).

Statistical analysis

Since we exposed parthenogenetic eggs to four test concentrations per pesticide and dormant eggs to five exposure concentrations, and the exposure concentrations differed per pesticide (based on previously published effect levels), we analysed the effects of each pesticide per egg type separately. Hatching success and developmental malformations of the embryos were related to exposure to each respective pesticide, using generalized linear models (GLM) with a logit-link function and binomial distribution, followed by sequential Bonferroni-correction to correct for multiple testing (Holm, 1979). Effect levels were estimated for all pesticides (EC_{50} values) using the DRC-package in R (Ritz and Streibig, 2005). Effects of the pesticides on timing of hatching (day of maximum hatching) were evaluated using one-way ANOVA´s followed by Tukey´s HSD post-hoc tests. Plate identity was taken into account by including it as a random blocking factor. As no significant differences were found between the blank and solvent controls, only results for the blank controls are presented. Statistical analysis were performed in R statistical software v3.0.2 (The R Foundation for Statistical Computing, 2013).

Results

Under control conditions, 94.9% of the parthenogenetic eggs hatched, all of them after three days and without any developmental malformations (Fig. 2). For the decapsulated dormant eggs, 89.6% hatched in the control treatment, with the peak of hatching after three days, and with 6.3% of the embryos showing developmental deformations (curved tail spine and/or underdeveloped second antennae; Fig. 2). All five pesticides significantly increased malformations in developing embryos of both parthenogenetic and dormant eggs (Fig. 2, Table 1). Low exposure levels mainly resulted in deformations of the tail spine (curved), while higher exposure concentrations caused severe (additional) malformations of the antennae and/or carapax (Fig. 1).

Table 1. Results of generalized linear model on effects of pesticides on hatching success and embryonic development (deformations) in both parthenogenetic and dormant eggs of *D. magna*, after a 10 day exposure during the hatching process.

Pesticide	Parthenogenetic eggs				Dormant eggs			
	Hatching		Deformations		Hatching		Deformations	
	Chi^2	p-value	Chi^2	p-value	Chi^2	p-value	Chi^2	p-value
Carbaryl	31.63	< 0.001 *	107.81	< 0.001 *	8.11	0.230	122.25	< 0.001 *
Fenoxycarb	2.16	0.707	134.01	< 0.001 *	50.08	< 0.001 *	219.55	< 0.001 *
Atrazine	0.93	0.921	22.93	< 0.001 *	12.49	0.052	88.22	< 0.001 *
Chlorpyrifos	9.46	0.051	113.31	< 0.001 *	3.44	0.752	134.28	< 0.001 *
Deltamethrin	2.20	0.699	132.48	< 0.001 *	5.12	0.529	158.65	< 0.001 *

The effect levels (EC_{50}) for developmental malformations of all tested pesticides are summarized in Table 2. Fenoxycarb induced malformations already at the lowest test concentration of 0.1 µg/L in both parthenogenetic and dormant eggs (Fig. 2C+D). However, most of these deformations were minor (curved tail spine), while severe deformations were abundant only after exposure to 1000 µg/L, in dormant eggs. Atrazine predominantly caused deformations of the tail spine in both egg types (EC_{50} for parthenogenetic eggs was above 100 µg/l and for dormant eggs 70.1 µg/L, respectively; Fig. 2E+F). The other three pesticides (carbaryl, chlorpyrifos and deltamethrin) all severely impacted embryonic development (deformations of carapax and second antennae) at the highest test concentrations, both in parthenogenetic and dormant eggs (Fig. 2A+B, Fig. 2G+H and Fig. 2I+J, respectively).

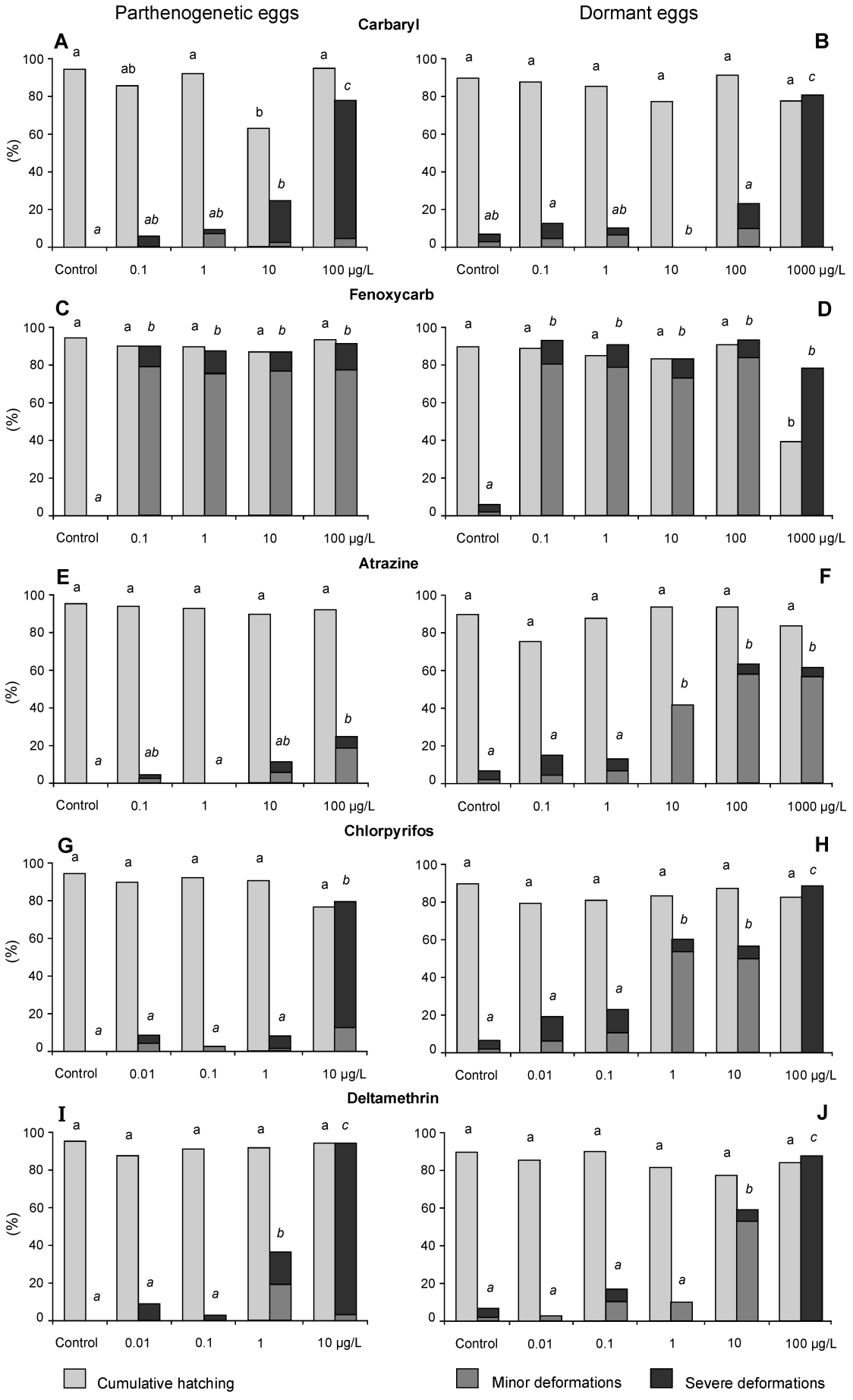
Table 2. Effect levels (EC_{50} with 95% confidence intervals) of pesticides on development of parthenogenetic and dormant eggs of *D. magna* (as observed in the present study), compared to effect levels on neonates (acute toxicity according to OECD 202, 2004), parthenogenetic reproduction (chronic toxicity according to OECD 211, 2012) and embryotoxicity of parthenogenetic eggs (from literature). All effect levels are expressed in µg/L.

Pesticide	**Parth.eggs**	**Dormant eggs**	**Neonates** (literature)	**Parth.repro** (literature)	**Parth. eggs** (literature)
	EC_{50}	EC_{50}	48h EC_{50}	21d NOEC	EC_x
Carbaryl	23.3 (9.6-37.3)	358.8 (199.0-518.6)	6-17 [1,2]	n.d. [1]	-
Fenoxycarb	< 0.1	< 0.1	500 [3]	0.002-3.2 [3]	EC_{80} = 3 [7]
Atrazine	> 100	70.1 (0-143.0)	3550 [4,5]	250 [4,5]	EC_{50} > 1 [8]
Chlorpyrifos	3.9 (1.6-6.2)	1.1 (0.2-1.9)	0.1-0.7 [4,5]	4.6 [4,5]	EC_{50} = 0.19 [9]
Deltamethrin	1.3 (0.6-2.0)	6.7 (2.8-10.6)	0.1-0.6 [4,6]	0.004 [4,6]	EC_5 = 0.3 [10]

[1] Coors et al., 2009; [2] EFSA, 2006; [3] EFSA, 2010; [4] PPDB; [5] Palma et al., 2008; [6] EC, 2003; [7] Mu and LeBlanc, 2004; [8] Palma et al., 2009; [9] Palma and Barbosa, 2011; [10] Toumi et al., 2013.

In decapsulated dormant eggs, dose-related effects on hatching success were observed after fenoxycarb exposure (reduction of 50.0% at 1000 µg/L; Fig. 2D, Table 1). In addition, fenoxycarb also significantly delayed hatching ($F = 21.88$, $p < 0.001$). At the highest test concentration of 1000 µg/L, the day of maximum hatching was 4.5 ± 0.2 (mean ± st.error), which was on average 1.5 days later than in the control treatments ($p < 0.001$). The other pesticides did not induce any significant effects on timing or success of hatching in dormant eggs ($p > 0.05$; Table 1). In parthenogenetic eggs, carbaryl had significant negative effects on hatching at 10 µg/L (Fig. 2A). However, no significant decrease in hatching was observed at the highest test concentration of 100 µg/L. The other pesticides did not show any significant effects on success and timing of hatching in parthenogenetic eggs ($p > 0.05$; Fig. 2E-J, Table 1).

Fig. 1 (right page) Effects of pesticide treatment on cumulative hatching percentage 10 days after exposure (light grey bars) and on the percentage of deformed embryos plus hatchlings (middle and dark grey bars, representing minor and severe deformations, respectively), of both parthenogenetic eggs (left panel) and dormant eggs (right panel) of *D. magna*. Parthenogenetic eggs were exposed to four concentrations of the respective pesticides, dormant eggs to an additional fifth concentration, a factor 10 higher. Distinct letters in the figures indicate significant differences among treatments for each pesticide for a specific variable ($n = 48$, $p < 0.05$, generalized linear model, followed by sequential Bonferroni-correction; see Table 1).


Parthenogenetic eggs
Dormant eggs
Carbaryl
A
B
Fenoxycarb
C
D
Atrazine
E
F
Chlorpyrifos
G
H
Deltamethrin
I
J
(%)
Control
0.01
0.1
1
10
100
1000 µg/L
Cumulative hatching
Minor deformations
Severe deformations

Discussion

In this study, we compared the sensitivity of asexual and sexual eggs from the same *Daphnia* population towards exposure to five model pesticides with a different mode of action. All pesticides impacted developmental processes in both parthenogenetic and dormant eggs, mainly resulting in embryonic malformations. We observed differences in sensitivity between the egg types and among pesticides tested, as illustrated by the differences in the severity of the effects and the effect concentrations.

Differences in sensitivity of parthenogenetic and dormant eggs

Overall there were no strong differences between parthenogenetic and dormant eggs, dormant eggs were not always less sensitive to pesticide exposure than parthenogenetic eggs, as we had hypothesized. In general, we observed a dose-related increase in embryonic deformations, but no significant impairment of hatching. For atrazine and chlorpyrifos, the effect levels (EC_{50}) for developmental malformations were lower in dormant eggs compared to parthenogenetic eggs. However, effects induced at these levels were mainly expressed as deformations of the tail spine. More severe effects (deformations of carapax and second antennae) were only observed at the highest tested exposure concentrations for all pesticides, except atrazine, and occurred at 10-fold lower concentrations in parthenogenetic eggs compared to dormant eggs. This might indicate that parthenogenetic embryos are exposed to higher internal pesticide levels than embryos of dormant eggs. Possibly, the additional membrane surrounding dormant embryos during the first developmental stages (Zaffagnini, 1987), partly protects the embryos against pesticide exposure (Navis et al., 2015; Chapter 3). However, at the highest test concentrations the pesticides still impacted embryonic development in dormant eggs, causing severe deformations (and for fenoxycarb, even a reduction in hatching success). Xenobiotics are known to cause teratogenicity and embryotoxicity in many different invertebrate species. The mechanisms involved are not yet completely understood, but could be related to oxidative stress, membrane alterations, modulations of energy supplies, enzyme inhibition and DNA alterations (Pašková et al., 2011).

Differences in effects of the model pesticides

In addition to differences in sensitivity between parthenogenetic and dormant eggs, we also observed differences in the effects of the five model pesticides. Parthenogenetic eggs were most sensitive to carbaryl, deltamethrin and fenoxycarb exposure. Fenoxycarb was the only pesticide to decrease hatching of dormant eggs. Atrazine had the lowest impact of all tested pesticides on development of both parthenogenetic and dormant eggs. Differences in impact of these model pesticides can be related to either their potential to bioconcentrate in the eggs or their capacity to interfere with embryonic development during the hatching process. While we do not have any information on the bioconcentration potential of atrazine in *D. magna* eggs, it has a low potential to bioconcentrate in *Daphnia* neonates (Nikkilä et al., 2001) and zebrafish embryos (BCF = 19; Wiegand et al., 2000).

Fenoxycarb, in contrast, is known to bioconcentrate in *D. magna* dormant eggs, especially during later developmental stages[1] (Navis et al., 2015; Chapter 3). Differences in effects of these pesticides might, however, also be caused by their different modes of action. Fenoxycarb mimics juvenile growth hormones in insects (EFSA, 2006) and is reported to disrupt endocrine processes in crustaceans (Oda et al., 2005; LeBlanc, 2007). Atrazine is a triazine herbicide, blocking photosynthesis in plants and reports on potential endocrine effects in *Daphnia* (induction of male offspring) are rather inconclusive (Olmstead and LeBlanc, 2003; Stoeckel, 2008; Palma et al., 2009a). Studies that include measurements of internal concentrations of pesticides and multiple representative compounds for each mode of action are needed to elucidate the mechanisms involved.

Methodological considerations

Four out of five model pesticides used in this study were selected based on previously reported effect levels on embryonic development of *D. magna* parthenogenetic eggs. For fenoxycarb, developmental malformations were reported in about 80% of the embryos at 3 µg/L (curved shell spine; Mu and LeBlanc, 2004). We found deformations at even lower test concentrations, with most deformations also being related to the tail spine. For chlorpyrifos and atrazine our results show effect levels above the ones previously reported (Palma et al., 2009a, 2009b; Palma and Barbosa, 2011). An EC_{50} for embryo toxicity of 0.2 µg/L was reported for chlorpyrifos (Palma et al., 2009a) and developmental deformations and arrest occurred at concentrations above 1 µg/L for atrazine (Palma and Barbosa, 2011). We observed severe deformations only at 10 µg/L for chlorpyrifos (66.7%) and significant effects on development at 100 µg/L for atrazine (23.7%). We did not observe any developmental arrest after exposure to either of the pesticides in parthenogenetic or dormant eggs. Toumi et al. (2013) reported 5.4% deformations in embryos after maternal exposure to 0.3 µg/L deltamethrin. We found significant effects on embryonic development at 1 µg/L (36.1% deformations), after direct embryonic exposure. Concentrations reported in present study, and several other studies (Palma et al., 2009a, 2009b; Palma and Barbosa, 2011) are nominal exposure concentrations, therefore direct comparison of effect levels should be interpreted with caution. Differences in results with previous studies, could reflect differences in sensitivity among *D. magna* populations and strains and slightly different test conditions (e.g. test medium, duration of experiment, maternal vs embryonic exposure).

Ecological relevance and the use of egg-based screening assays

Currently, in environmental risk assessment, safety factors are used to account for inter- and intra-species variation when calculating safe levels for the environment (PNEC; Walker, 2014). This includes variation between different stages of the life cycle.

[1] During development of an analytical method in order to determine pesticide concentrations inside *D. magna* dormant eggs (as described in detail in Chapter 3), internal egg concentrations of both carbaryl and fenoxycarb were determined. After exposure to the highest test concentrations used in the experiments (4 mg/L fenoxycarb, and 5 mg/L carbaryl, respectively) internal concentrations were 220.0 ng fenoxycarb/100 eggs and 5.0 ng carbaryl/100 eggs (average, n = 3). This clearly indicates a difference in the potential to bioconcentrate in dormant eggs between the two model pesticides.

When comparing effect levels from our study with effect levels from traditional ecotoxicological assays using *D. magna* neonates (according to OECD 202; OECD, 2004 and OECD 211; OECD, 2012), we can conclude that there is not one life stage that appears most sensitive to all pesticides tested (Table 2). Instead this seems to be depending on the type of pesticide (mode of action).

Screening assays using both parthenogenetic and dormant eggs, like in the present study, can give a first impression of potential embryotoxic effects of various types of chemicals. However, the direct environmental relevance of these simple, short-term tests is limited since exposure is maximized during the hatching process (for dormant eggs, the protective ephippium was removed) and takes place under standardized laboratory conditions (fixed temperature and photo-regime), with eggs placed individually in the wells of multiwell plates. Concentrations tested here span a wide range, with the highest test concentrations above measured or predicted environmental levels (e.g. Norris et al., 1983; Walters et al., 2003; EFSA, 2010). These levels could be compared to pulse exposure in spring, shortly after pesticide application in the field, which could coincide with the spring hatching peak in temperate lakes (Alekseev and Lampert, 2001). Observed embryotoxic effects can have impacts on hatchling survival and life-history traits when tested under laboratory conditions (Navis et al., 2013; Moest et al., 2015; Chapter 2) However, it remains to be tested whether the observed effects are similar when pesticide exposure takes place under more realistic environmental conditions, including biotic interactions and using different exposure pathways.

Hatching experiments using isolated parthenogenetic and dormant eggs can be an effective screening method, which could be useful for ranking chemicals and comparing effect levels with other stages of the life-cycle in the model organism *D. magna*. In a broader, ecological context, more research into ecotoxicological effects affecting both reproductive phases (sexual vs asexual reproduction) is needed. Since there are many different taxa that combine sexual and asexual reproduction in their lifecycle, such as rotifers, cnidarians, bryozoans, plants, protists and many parasites (De Meester et al., 2004; Decaestecker et al., 2009).

Acknowledgements

This research was funded by a Ph.D. grant of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen). The authors would like to thank Laetitia Beullens, Thomas Nicolaï and Adinda Putman for their practical assistance during the laboratory experiments. And Ineke Swillen for her valuable insights during preparation of the manuscript.

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CHAPTER 2

Pesticide exposure impacts not only hatching of dormant eggs, but also hatchling survival and performance in the water flea *Daphnia magna*

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Ecotoxicology (2013) 22(5):803-14

Abstract

Laboratory ecotoxicity tests and biomonitoring in aquatic systems are currently based on the active component of invertebrate communities. Even though dormant egg banks are crucial for the long term survival and community dynamics of many aquatic organisms, the effects of anthropogenic activities on dormant egg bank dynamics have rarely been studied. In this study we investigated the effects of two pesticides with a different mode of action (carbaryl and fenoxycarb) on hatching of *Daphnia magna* dormant eggs (ephippia) as well as on survival, growth and reproduction of the hatched neonates. Dormant eggs were exposed to the pesticides simultaneously to incubation under conditions that induce hatching (long daylight and 20°C). Carbaryl had no negative effects on embryonic development or hatching rate up to concentrations almost 1000 times the median effect concentration (EC_{50}) of neonate survival in acute tests. Fenoxycarb, however, had a significant dose-related effect by delaying or completely stopping the hatching process and caused severe abnormalities in developing individuals. Both pesticides had significant negative effects on survival and reproduction of the hatchlings. These results indicate that, in addition to inducing mortality of active individuals, pesticides can affect zooplankton communities by altering hatching dynamics and life history traits of hatched individuals. We briefly discuss how such pollution induced changes in the benthic-pelagic coupling could translate into trans-generational effects impacting ecological and evolutionary dynamics.

Introduction

Many invertebrate species living in permanent and temporary standing waters produce dormant stages to survive unfavourable environmental conditions, such as drought, low oxygen concentrations, food limitation, crowding and the presence of predators (Hairston et al., 1990; Alekseev and Starobogatov, 1996; Brendonck et al., 1998; Slusarczyk et al., 1999, 2005). Dormant eggs accumulate over the years in the sediment to form a dormant egg bank, analogous to plant seed banks (De Stasio, 1989; Hairston and Cáceres, 1996), with each growing season only a fraction of the dormant eggs hatching. Through this benthic-pelagic coupling, events in the dormant phase can affect the active, aquatic phase and vice versa (Cáceres and Hairston, 1998; Gyllström and Hansson, 2004). Zooplankton dormant eggs can remain viable for hundreds of years (Hairston et al., 1995; Cáceres, 1998). With densities in the sediment ranging between 10^3-10^6 eggs/m^2 (Hairston, 1996; Brendonck and De Meester, 2003; Vandekerkhove et al., 2005a), the dormant fraction is far from negligible and may strongly impact ecological and evolutionary dynamics (Gyllström and Hansson, 2004). When environmental conditions fluctuate, egg banks can function as a reservoir of species and genetic diversity through a mechanism called the "storage effect" (Chesson and Warner, 1981; Cáceres, 1997). Dormant egg banks integrate genetic variation that has accumulated over several growing seasons (Ellner and Hairston, 1994; De Meester et al., 2006) thereby increasing the evolutionary potential of a population (Hairston and De Stasio, 1988; Hedrick, 1995; Brendonck and De Meester, 2003). Despite its importance in ecological and evolutionary processes (reviewed in Brendonck and De Meester, 2003), dormant egg bank dynamics are rarely included in zooplankton population and community studies (Cáceres, 1998; Hairston et al., 2000) and there is almost no information available on the effects of pollution on dormant egg bank dynamics.

Daphnia magna, a well established model organism and standard test species in ecotoxicology (Walker, 2001), reproduces by cyclical parthenogenesis. Production of sexual, dormant encapsulated eggs (ephippia) is triggered by conditions announcing or associated with an unfavorable environment (Hebert, 1978; Decaestecker et al., 2009). Standard ecotoxicity tests with *Daphnia* (OECD TG 202, 2004; OECD TG 211, 2012) generally focus on the effects of chemicals on the asexual part of the reproduction cycle, screening clonal lineages. However, chemical compounds can interfere with the life cycle of *Daphnia* through a number of different pathways: they can i) alter the hatching rate of dormant eggs, either by impacting the embryos (Fig. 1, number 1) or by changing the response to hatching cues (Fig. 1, number 2) (Angeler et al., 2005; Raikow et al., 2006, 2007); ii) affect survival and growth of hatched individuals (Fig. 1, number 3); iii) change the fitness and reproductive traits of parthenogenetic females (Fig. 1, number 4) (Barata et al., 2007; Hassold and Backhaus, 2009; Jansen et al., 2011); iv) change offspring sex ratio (Fig. 1, number 5) (Dodson et al., 1999; Olmstead and LeBlanc, 2003; Tatarazako and Oda, 2007; Palma et al., 2009); and v) affect timing and amount of dormant egg production (Fig. 1, number 6) (Shurin and Dodson, 1997; Olmstead and LeBlanc, 2001; Ignace et al., 2011).

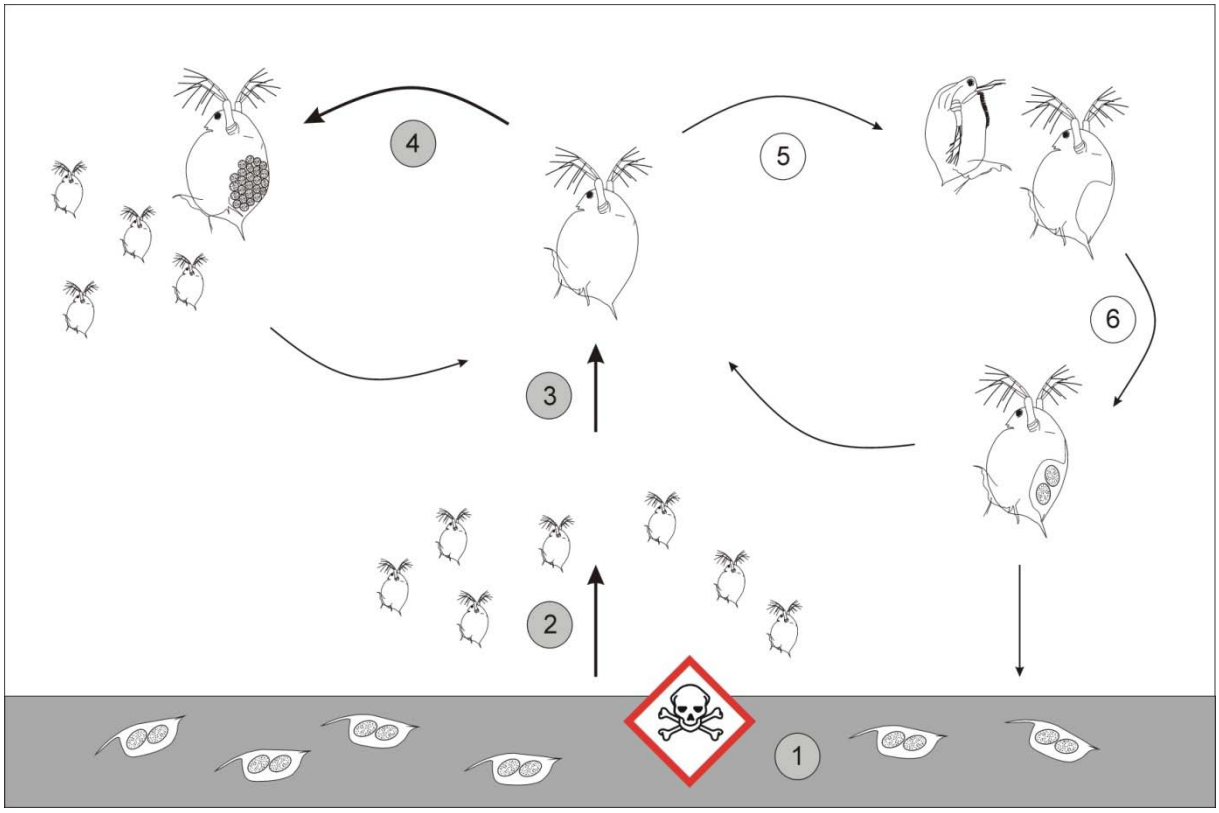

Fig. 1 Toxicants can have an effect on different stages of the *Daphnia* reproduction cycle: 1) direct mortality of the dormant eggs; 2) effects on hatching characteristics of the dormant eggs; 3) effects on growth; 4) effects on asexual reproduction; 5) effects on sexual reproduction (through changes in sex ratio) and 6) effects on dormant egg production. This study is focused on pathways 1, 2, 3 and 4, following toxicant exposure of the dormant eggs.

Little information is available on the effects of chemical exposure on survival and hatching of zooplankton dormant eggs in general, and in *Daphnia* more specifically. A few studies evaluated the efficiency of ship ballast tank treatments, in order to avoid introduction of exotic/invasive species through their dormant stages. The biocides sodium hypochlorite and menadione (SeaKleen) affected hatching success of *D. mendotae* dormant eggs, but effect levels were higher compared to other *D. mendotae* life stages (Raikow et al., 2006, 2007). Angeler et al. (2005) tested for effects of fire retardant treatments and found a significant negative effect of Fire Trol 943 on emergence of *D. curvirostris* dormant eggs from wetland sediments. For rotifers, Marcial et al. (2005) found that diazinon, fenitrothion, methoprene and isoprothiolane affected hatching of *Brachionus plicatilis* dormant eggs at concentrations 2-40 times lower than concentrations affecting population growth, mixis and fertilization endpoints. Marcial and Hagiwara (2007) discovered that *B. plicatilis* hatching rates were severely affected by diazinon, but only when rotifers were exposed during dormant egg production or shortly afterwards. Copepod dormant eggs (*Acartia pacifica)* were much more sensitive to metals than benthic adults (Jiang et al., 2007). The results of different studies using *Artemia* cysts, however, appear to be inconclusive. Varó et al. (2006) and Sarabia et al. (2003, 2008) reported no adverse effects of metal and pesticide exposure on hatching of *Artemia* cysts. Bagshaw et al. (1986) and Rafiee et al. (1986), on the contrary, reported that hatching of dormant eggs was more sensitive to heavy metal exposure than hatched individuals.

In this study we have tested for acute and chronic effects of pesticide exposure on dormant eggs of *D. magna*. Therefore we have conducted two series of experiments: in a first series we focused on the effects of pesticides on embryonic development and hatching characteristics of the dormant eggs (Fig. 1, number 1+2), in a second experiment we tested whether exposure to pesticides could have long term effects on survival and life history characteristics of the hatched neonates (Fig. 1, number 3+4). For our experiments, we selected two model pesticides with a different mode of action: carbaryl and fenoxycarb. Carbaryl is a carbamate insecticide, which causes overstimulation of the nervous system by binding to acetylcholine esterase (Walker et al., 2001), and has a very high acute toxicity for crustaceans (EFSA, 2006). Fenoxycarb, on the other hand, is an insect growth regulator, which is known to disturb the endocrine system in crustaceans (LeBlanc, 2007), by changing offspring sex ratio (Tatarazako and Oda, 2007) and causing developmental malformations in parthenogenetic eggs (Mu and LeBlanc, 2004). Our working hypothesis is that exposure to pesticides, if it coincides with incubation under optimal hatching conditions, might impact embryonic development and hatching rate of the dormant eggs, as well as life history characteristics of the hatched neonates. We expect the effects to be different for the two model pesticides, with a more pronounced effect of fenoxycarb, known to affect developmental processes in invertebrates.

Material and Methods

Daphnia magna *ephippia*

Daphnia magna ephippia from two different sources were used; 1) from a field population "Langerodevijver" (LRV: Korbeek-Dijle, Belgium) and 2) from a standardized laboratory culture (MBT: MicroBioTests Inc., Mariakerke, Belgium). The LRV field population was selected because it is located in a rather pristine environment (natural reserve "Doode Bemde": Orsini et al., 2012) and has a high density of *D. magna* ephippia (Rousseaux, 2011). The dormant egg bank was sampled in the winter period (December 2011) since the dormant egg bank then has its maximum size and eggs are in diapause. A plexiglass sediment corer (diameter 5 cm, length 1 m) was used for sampling at 10 locations in the open water zone of the lake. Only the top 5-10 centimeters of the cores (active egg bank: Cáceres & Hairston, 1998) were retained. Pooled sediment samples were sieved over 1 mm and 250 µm sieves and *D. magna* ephippia were isolated and stored for at least one month in the dark at 4°C (according to Vandekerkhove et al., 2005b). MBT ephippia are internationally used in standard acute toxicity assays and hatched neonates have a sensitivity similar to that of parthenogenetic offspring from lab cultures (Persoone et al., 2009). All ephippia were mechanically decapsulated with metal tweezers shortly before the start of the experiments and only healthy eggs were used.

Effects on embryonic development and hatching characteristics

In a first range-finding experiment a wide range of carbaryl and fenoxycarb concentrations were applied to find effect levels on hatching characteristics of LRV dormant eggs. Carbaryl (1-naphthyl methylcarbamate, CAS no. 63-25-2, 99.8% purity, Sigma-Aldrich, Germany) and fenoxycarb (ethyl 2-(4-phenoxyphenoxy)ethylcarbamate, CAS no. 72490-01-8, 99.6% purity, Sigma-Aldrich, Germany) were dissolved in absolute ethanol (purity min. 99.8%, VWR International, France). The concentration of ethanol was the same in all treatment solutions and in the solvent control (0.005% ethanol). Exposure solutions were freshly prepared with final concentrations of 5, 50, 500, 1000 and 5000 µg/L for carbaryl and 0.5, 5, 50, 500, and 5000 µg/L for fenoxycarb. These concentrations were chosen based on the sensitivity of *Daphnia* neonates as established in standard acute ecotoxicological tests (EFSA, 2006; Coors et al., 2009; EFSA, 2010) and previous preliminary experiments (S. Navis, unpublished data). For carbaryl we choose concentrations up to 1000 times the EC_{50} (6-17 µg/L) for neonate immobilisation. For fenoxycarb we choose a range of concentrations from 1000 times below the EC_{50} for neonate immobilisation (500-600 µg/L) up to 10 times above, because we expected effects on embryonic development at low concentrations from earlier work by Mu and LeBlanc (2004). Although no longer registered for use in Europe, carbaryl is still widely used in amongst others, the United States, Canada and Australia. Observed field concentrations for carbaryl are generally low, ranging from below the detection limit up to 5.5 µg/L (US EPA, 2003). However, peak concentrations of 1.74 mg/L and 4.8 mg/L have been reported in periods immediately following application (Norris et al., 1983; Bridges et al., 1999; Walters et al., 2003). Tested carbaryl concentrations are thus in the range of measured environmental concentrations. For fenoxycarb no field measurements of small water bodies were found in literature. Environmental concentrations (PEC) in surface waters immediately after application of 87.6 µg/L have been predicted (FOCUS 1 scenario; EFSA, 2010).

Dormant eggs were placed individually in the wells of a 24-well microtiter plate (polystyrene, non coated, sterile plates, Greiner Bio-One GmbH) containing 2 mL of exposure medium, prepared in artificial freshwater (ADaM; Klüttgen et al., 1994). Per treatment 48 dormant eggs (replicates) were used. Treatments were randomly allocated to plates in such a way that each treatment was allocated to four randomly assigned half multiwell plates. The influence of plate identity was taken into account by including it as a random blocking factor in the statistical analyses. Plates were incubated for 10 days at 20±2°C in a light:dark regime of 18:6h, to simulate spring conditions. Dormant eggs were checked daily for hatching. In a second experiment, a more narrow range of fenoxycarb concentrations (50, 125, 250, 500, 1000, 2000 and 4000 µg/L) was selected, based on the results of the range-finding experiment, to test for effects on both embryonic development and hatching characteristics of the dormant eggs. This was not done for carbaryl, since even the highest test concentration in the range-finding experiment did not have a significant effect. Test procedures were identical to the previous experiment; plates were incubated for 10 days and dormant eggs were checked daily for hatching. In addition, during day 3-7 of the experiment, embryonic development and morphological abnormalities were monitored using a digital camera (Olympus Colour view III) attached to an Olympus CKX41 inverted microscope (magnification 10x).

For this series of experiments, developmental abnormalities of the embryos as well as hatching rate (cumulative hatching %) related to pesticide exposure were statistically evaluated using generalized linear models (GLM) with a logit-link function and binomial distribution, followed by sequential Bonferroni-correction (Holm, 1979). Effects of the pesticides on timing of hatching (day of maximum hatching) were evaluated for the two pesticides separately using one-way ANOVA´s followed by Tukey´s HSD post-hoc tests. Concentration-response curves and effect levels (EC_{50} values) were estimated using the DRC-package in R (Ritz and Streibig, 2005).

Life cycle toxicity: from hatching to reproduction

In a second series of experiments, the effects of carbaryl and fenoxycarb exposure, on growth, maturation and reproductive capacity of *D. magna* were assessed by adapting standard methods for chronic toxicity tests (OECD TG 211; OECD, 2012). Based on results of the first series of experiments, three test concentrations for each pesticide were selected: 50, 500 and 5000 µg/L for carbaryl and 500, 750 and 1000 µg/L for fenoxycarb. Dormant eggs from both LRV and MBT were used in a full factorial design: 2 populations * 2 pesticides * 4 pesticide concentrations (incl. control) = 16 treatments. Two controls were included: a blank control containing only ADaM-water and a solvent control with 0.005% ethanol (equal to the ethanol concentration in all exposure solutions). Dormant eggs were individually placed in 24-well microtiter plates, with a total of 48 eggs (replicates) per treatment and each treatment was randomly divided over four half plates. To ensure that exposure to the pesticides could only occur during the hatching process and not after the organisms had hatched, the eggs were transferred into new microtiter plates with 2 mL fresh ADaM-water at the end of the second day of exposure (before any eggs had hatched) and rinsed once more to ensure that no exposure medium could be present in the wells. Hatching was checked daily and from each treatment 15 randomly selected hatchlings (replicates) from the day of maximum hatching were transferred individually into 100 mL glass beakers with ADaM-water. Daphnids were fed daily with *Scenedesmus obliquus* ($1*10^5$ cells/mL), and medium was renewed every two days. All individuals were monitored daily for at least 21 days, or until release of the second brood. Offspring were counted and removed from the test vessels daily. Survival, age at maturation, age at release of the first and second clutch and clutch size of first and second brood were recorded. The performance (r) was computed iteratively from the Euler-Lotka equation (Begon et al., 2005) and provides an estimate of the intrinsic rate of increase under the assumption of the absence of mortality:

$$\sum_{x=0}^{\infty} e^{-rx}\, lx\, mx = 1$$

where l_x represents the proportion of individuals surviving to age x (days; here we calculated performance only for surviving individuals so l_x is assumed to be 1) and m_x is the number of juveniles produced by the individual between the ages x and x + 1. Performance was calculated only for the first brood, since some individuals died before producing a second brood.

To monitor growth, body size of all experimental organisms was monitored by photographing each individual directly after hatching, after 10 and 21 days as well as at maturation and at time of first and second reproduction. In addition, from each individual three neonates per brood were photographed. All images were recorded using a digital camera (Olympus Colour view III) attached to an Olympus BX50 microscope (magnification adults 2x, neonates 4x) and processed later for measurements of body length (from top of the eye to base of the tail spine). Main effect of pesticide and population (origin of the dormant eggs) and the pesticide x population interaction effect on survival of the hatched neonates, were tested for each pesticide separately using generalized linear models (GLM) with a logit-link function and binomial distribution, followed by a sequential Bonferroni-correction. Effects of pesticide and population origin on life history traits (growth and reproduction) were assessed for the two pesticides separately using two-way ANOVA´s followed by Tukey´s HSD post-hoc tests. All statistical analysis were performed in R statistical software v2.15.0 (R Development Core Team, 2012). All pesticide concentrations mentioned are initial nominal concentrations. In all experiments a blank control and a solvent control treatment were included. If the results of both control treatments were not statistically different, only results from the blank controls were reported.

Results

Carbaryl

Exposure of LRV dormant eggs to carbaryl concentrations up to 5000 µg/L had no significant effects on hatching characteristics. Cumulative hatching percentages after 10 days ranged from 52.1 – 81.3%, but there was no concentration-response relationship (Chi^2 = 10.12; df= 5; p = 0.072). The NOEC for hatching in the presence of carbaryl is therefore ≥ 5000 µg/L. Carbaryl did have a significant negative impact on survival of the hatched individuals (Fig. 3A+C). For MBT dormant eggs, survival in the control treatment was 86.7% and all three tested carbaryl concentrations resulted in significantly reduced survival compared to the control (Fig. 3A). For LRV dormant eggs, survival in the control treatment was 53.3% and only the highest tested carbaryl concentration of 5000 µg/ had a significant negative impact on survival (Fig. 3C).

Carbaryl also significantly affected other life history traits (Table 1). For MBT, all three tested carbaryl concentrations caused a significant reduction in performance (r), a higher age at maturity and at release of the first and second brood, a lower clutch size (first and second brood) and a smaller body size (measured after 21 days, corrected for body size directly after hatching) (Fig. 4). No significant effects of carbaryl on offspring body size of the first brood were detected, but offspring of the second brood was significantly smaller in carbaryl treatments compared to the controls. For LRV, only significant negative effects were found for age at first reproduction at the highest test concentration. Other measured traits did not differ significantly from the controls. The LRV population showed very large variation in responses within treatments, also for the controls, especially regarding clutch sizes. In addition, survival at the highest pesticide concentrations was very low (n = 3), resulting in reduced statistical power.

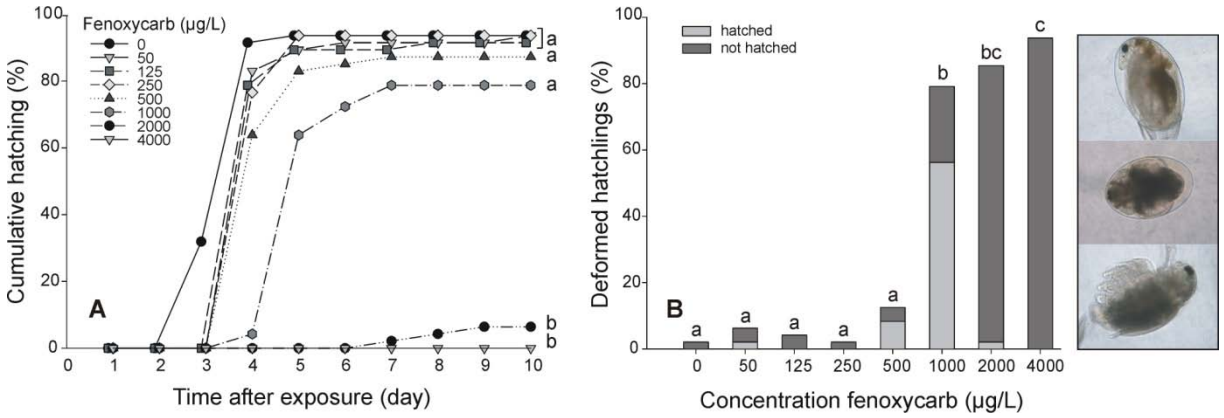

Fig. 2 Effects of fenoxycarb treatments on A) cumulative hatching percentage during 10 days after exposure and B) the percentage of deformed hatchlings (n = 48). Fenoxycarb both decreased the hatching success and increased the percentage of deformations in a dose-dependent manner. Distinct letters in the figures indicate significant differences among treatments ($p \leq 0.05$, generalized linear model, followed by sequential Bonferroni-correction). The photographs represent (from top to bottom): a normal developing dormant egg from the control treatment; an embryo with developmental malformations; and a deformed hatchling.

Fenoxycarb

Fenoxycarb had a significant negative effect on hatching of *D. magna* dormant eggs (LRV). In the preliminary range-finding experiment, hatching at the highest tested fenoxycarb concentration (5000 µg/L) was completely inhibited ($Chi^2 = 79.47$; df= 5; $p < 0.001$). In the final experiment, hatching rate was significantly negatively impacted at concentrations 2000 and 4000 µg/L (cumulative hatching was respectively 6.3 and 0%; $Chi^2 = 267.78$; df= 7; $p < 0.001$) (Fig. 2A) and an EC_{50} for hatching of 1300 µg/L was estimated. Timing of hatching was delayed at concentrations above 250 µg/L ($F = 56.28$; df = 6; $p < 0.001$). In the control treatment, the peak of hatching occurred at day 3, while in the fenoxycarb treatments maximum hatching occurred at day 4-7.

Analysis of the photographs of the developing embryos revealed that fenoxycarb caused developmental abnormalities (Fig. 2B), including poorly developed second antennae, morphological deformations of the tail spine, carapace and compound eye. Embryonic development was severely altered or arrested at concentrations of 1000, 2000 and 4000 µg/L fenoxycarb, with abnormalities observed in respectively 79.2, 85.4 and 93.8% of the embryos. At 1000 µg/L, 56.3 out of 79.2% abnormally developed embryos still hatched. At higher test concentrations almost none of the deformed embryos was able to hatch (2.1 and 0% at respectively 2000 and 4000 µg/L).

Fenoxycarb had a significant negative impact on survival of the hatched individuals. For MBT, all three tested concentrations resulted in significantly reduced survival compared to the control treatments (Fig. 3B). For LRV dormant eggs, only the highest tested concentration of 1000 µg/L had a significant negative impact on survival (Fig. 3D).

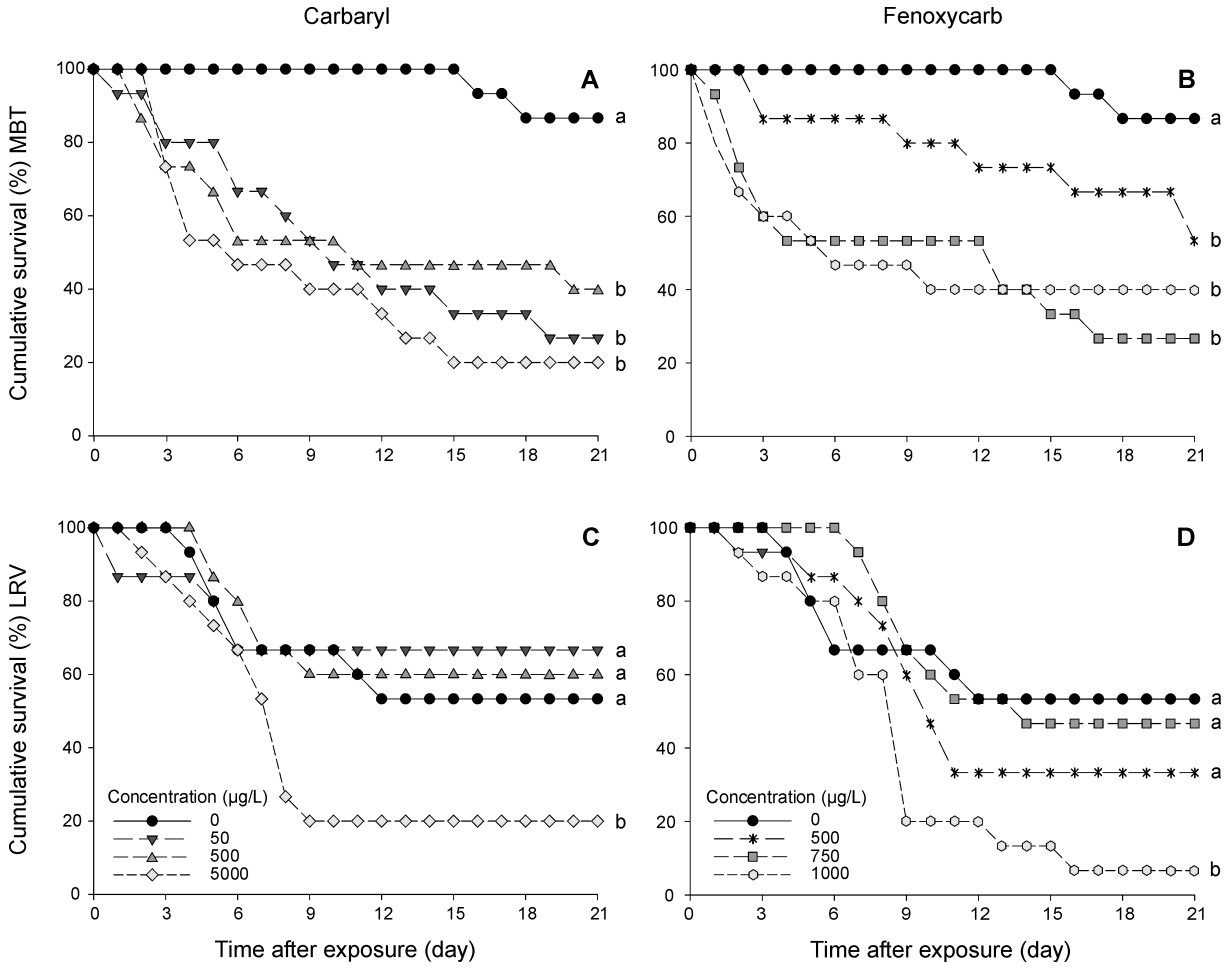

Fig. 3 Cumulative survival percentage of hatched individuals from different pesticide treatments (n = 15, replicates) during the 21 days of the experiment: A) carbaryl, hatched from dormant eggs produced in the laboratory under controlled conditions (MBT); B) fenoxycarb, hatched from MBT; C) carbaryl, hatched from a natural source of dormant eggs (LRV); D) fenoxycarb, hatched from LRV. Distinct letters in the figures indicate significant differences among treatments ($p \leq 0.05$, generalized linear model, followed by sequential Bonferroni-correction).

Fenoxycarb also had a significant negative effect on life history traits of the surviving individuals (Table 1). For MBT, all three test concentrations caused a decrease in performance (r), a higher age at maturity and at release of the first and second brood and a lower clutch size (first and second brood) (Fig. 4). For the highest concentration there was also a negative effect on growth of the experimental organisms. Again no significant effects on offspring body size (first and second brood) were detected. For LRV, all three fenoxycarb concentrations had significant negative effects on performance and fenoxycarb 750 µg/L had a significant negative effect on age at first brood, but no significant effects on other life history traits were detected.

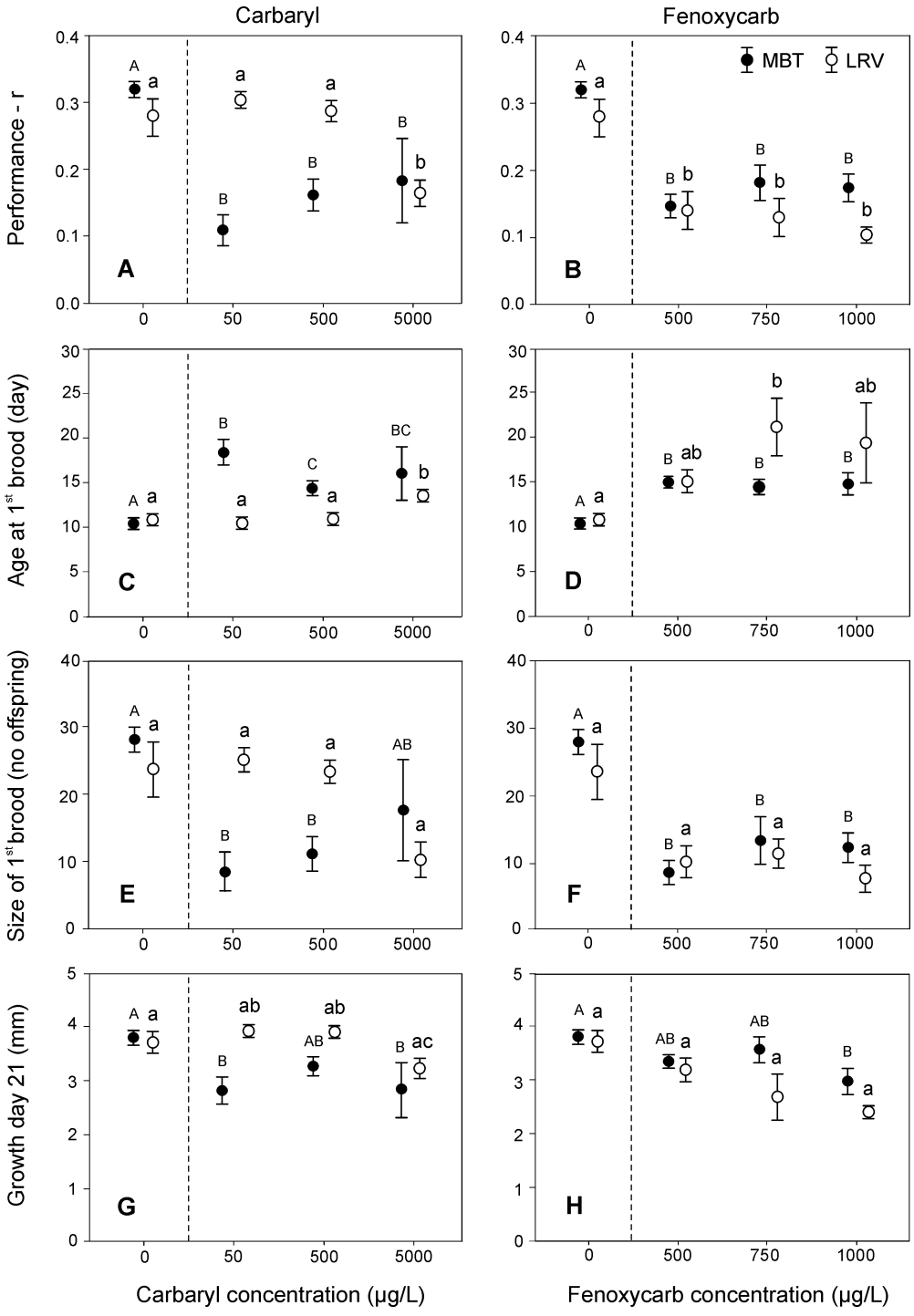

Fig. 4 Effects of carbaryl (carb) and fenoxycarb (fnx) exposure on growth and reproduction (average ± 1 SE) of *D. magna* hatchlings (number of replicates (n) depends on survival of the hatched individuals, in panels A-F from left to right and per population; MBT control: 15, carb50: 5, carb500: 5, carb5000: 2, and fnx500: 11, fnx750: 4, fnx1000: 5, LRV control: 9, carb50: 10, carb500: 9, carb5000: 3, fnx500: 5, fnx750: 6, fnx1000: 2, and in panels G-H; MBT control: 13, carb50: 4, carb500: 7, carb5000: 3, and fnx500: 10, fnx750: 4, fnx1000: 6 and for LRV control: 8, carb50: 10, carb500: 9, carb5000: 3, fnx500: 5, fnx750: 7, fnx1000: 1). Letters above each result indicate significant differences among treatments ($p \leq 0.05$, 2 -way ANOVA, Tukey´s HSD post-hoc test; capital letters are used for the MBT population and lower case for the LRV population).

Table 1 Results of the 2-way ANOVA on the life history traits of *D. magna* for the two model pesticides

	Concentration (df = 3)		**Population** (df = 1)		**Conc * population** (df = 3)	
	F	P	F	P	F	P
Carbaryl						
Performance (r)	10.34	< 0.001 *	18.75	< 0.001 *	15.40	< 0.001 *
Age at maturity	11.65	< 0.001 *	34.61	< 0.001 *	9.63	< 0.001 *
Age at 1st brood	12.22	< 0.001 *	37.44	< 0.001 *	18.88	< 0.001 *
Age at 2nd brood	5.42	0.003 *	24.95	< 0.001 *	11.94	< 0.001 *
Size of 1st brood	6.34	< 0.001 *	5.62	0.022 *	7.58	< 0.001 *
Size of 2nd brood	2.43	0.077	8.16	0.006 *	4.65	0.006 *
Body size at hatching	4.69	0.004 *	3.49	0.064	0.78	0.509
Growth hatching - day 11	24.13	< 0.001 *	59.08	< 0.001 *	16.97	< 0.001 *
Growth hatching - day 21	4.30	0.009 *	11.93	0.001 *	4.67	0.006 *
Body size of offspring 1st brood	0.10	0.960	0.09	0.759	0.84	0.477
Body size of offspring 2nd brood	5.88	0.002 *	12.60	< 0.001 *	3.41	0.025 *
Fenoxycarb						
Performance (r)	30.53	< 0.001 *	4.25	0.045 *	0.49	0.691
Age at maturity	16.24	< 0.001 *	5.59	0.022 *	3.35	0.026 *
Age at 1st brood	17.20	< 0.001 *	6.53	0.014 *	2.94	0.042 *
Age at 2nd brood	13.59	< 0.001 *	0.82	0.371	1.43	0.251
Size of 1st brood	20.43	< 0.001 *	1.46	0.234	0.44	0.723
Size of 2nd brood	5.38	0.003 *	0.40	0.529	0.17	0.844
Body size at hatching	8.42	< 0.001 *	31.87	< 0.001 *	0.96	0.414
Growth hatching - day 11	24.36	< 0.001 *	2.80	0.099	0.60	0.617
Growth hatching - day 21	5.93	0.002 *	3.41	0.071	1.14	0.343
Body size of offspring 1st brood	0.32	0.813	0.01	0.923	1.15	0.341
Body size of offspring 2nd brood	4.25	0.010 *	2.84	0.099	0.50	0.610

Discussion

Our results reveal the effects of two model pesticides with a different mode of action on hatching characteristics of *D. magna* dormant eggs and on subsequent survival and life history characteristics of hatched individuals. Overall, our results indicate that dormant eggs are quite insensitive to carbaryl with respect to hatching rates, while they are influenced by fenoxycarb at relatively moderate concentrations. Survival and performance of hatchlings were impacted by both pesticides. We discuss these results and highlight the possible impact of the observed effects on the development and dynamics of zooplankton populations in natural systems.

Impact of pesticides on hatching, growth and reproduction

Carbaryl did not have a significant negative impact on hatching up to the highest tested concentration (NOEC ≥ 5000 µg/L). This is a striking observation as acute effect levels of neonate immobilisation reported for carbaryl range between 6-17 µg/L (EFSA, 2006; Coors et al., 2009), i.e. 300 to almost 1000 times lower concentrations than used in our experiment. This indicates that for carbaryl, hatching and development of *D. magna* dormant eggs is a much less sensitive endpoint than neonate immobilisation, as used in standard acute toxicity tests.

For fenoxycarb, however, we observed clear dose-related effects on dormant eggs, resulting in a delay in hatching or even complete absence of hatching. The EC_{50} of fenoxycarb for hatching is 1300 µg/L, which is about twice as high as the acute effect level for neonate immobilisation of 500-600 µg/L (EFSA, 2010). Fenoxycarb also caused severe abnormalities in developing individuals at 1000 µg/L or higher test concentrations, which is again above the EC_{50} for neonate immobilisation. Fenoxycarb is known to cause similar malformations in parthenogenetic eggs, but at much lower (1000 times) concentrations than found in our study (Mu and LeBlanc, 2004). Overall, we can conclude that, for both model pesticides, *D. magna* was less sensitive when pesticide exposure occurs during embryonic development and hatching than during other life stages. A possible explanation for the lower sensitivity at this stage may be that dormant eggs are better protected against penetration of pesticides. Besides the protective envelope, which was removed in our experiments through decapsulation, they have thick, multi-layered egg membranes which enable them to endure extreme physical conditions like freezing, desiccation and bird digestion enzymes (Mellors, 1975; Frisch et al, 2007). Varó et al. (2006) demonstrated that the chorion surrounding cysts of *Artemia* can act as a barrier retaining the majority of the toxicant. However, our results do indicate that the pesticides were able to enter the dormant eggs, as both pesticides had significant negative effects on hatchling survival, growth and reproduction, even though the eggs were only exposed during the first two days of the hatching process and were transferred to clean medium before hatching occurred. Our observation that effects were only observed at relatively high concentrations, corresponding to environmental concentrations measured shortly after application, may reflect that the concentration of the pesticides inside the eggs was relatively low compared to the nominal concentrations used in the exposure medium[1]. This might also explain why carbaryl had no significant effects on hatching characteristics up to the highest concentration tested, but did have significant chronic effects, that typically occur at lower exposure levels. Moreover, this study is the first to demonstrate that exposure to toxicants in the dormant phase cannot only affect hatching rates, but also results in reduced reproductive rates of hatched individuals.

The large differences between carbaryl and fenoxycarb regarding their effects on hatching and life history characteristics of *D. magna*, can at least partly be explained by their different mode of action. Carbaryl is a carbamate insecticide with neurotoxic activity, causing overstimulation of the nervous system (Walker et al., 2001). Fenoxycarb, on the other hand, is a juvenile hormone analogue and a very potent methyl farnesoate agonist (Tatarazako et al., 2003; Oda et al., 2005; Wang et al., 2005) that is able to induce the production of male neonates in *D. magna* at concentrations as low as 100 ng/L (Tatarazako and Oda, 2007). Fenoxycarb can also disrupt ecdysteriod-regulated aspects of embryo development in parthenogenetic eggs, but at levels ten times higher compared to effects on offspring sex ratio (Mu and LeBlanc, 2004).

[1] During development of an analytical method in order to determine pesticide concentrations inside *D. magna* dormant eggs (as described in detail in Chapter 3), internal egg concentrations of both carbaryl and fenoxycarb were determined. After exposure to the highest test concentrations used in the experiments (4 mg/L fenoxycarb, and 5 mg/L carbaryl, respectively) internal concentrations were 220.0 ng fenoxycarb/100 eggs and 5.0 ng carbaryl/100 eggs (average, n = 3). This clearly indicates a difference in the potential to bioconcentrate in dormant eggs between the two model pesticides.

Due to their different mode of action, fenoxycarb was expected to interfere with embryonic development while carbaryl was expected to have an effect mainly when the nervous system has developed. This was largely confirmed in our study. Despite the importance of dormant egg banks for the persistence of zooplankton communities in general and *Daphnia* populations in particular (Brendonck and De Meester, 2003), little information is available on the effects of chemical exposure on survival and hatching of dormant eggs. Significant negative effects of toxicants on hatching of *Daphnia* dormant eggs have been reported previously in a few studies (Angeler et al., 2005; Raikow et al., 2006, 2007). Our findings are in agreement with the results of Raikow et al. (2006, 2007), who observed that the biocide SeaKlean had significant negative effects on hatching of *D. mendotae* dormant eggs, but with a ten times lower sensitivity compared to neonate mortality (Song et al., 2011). Studies using dormant eggs of other zooplankton species (mainly rotifers and *Artemia*) gave variable results; ranging from no significant adverse effects on hatching rates (Sarabia et al., 2003, 2008; Varó et al., 2006; Marcial and Hagiwara, 2007), to effects on unhatched embryos at concentrations below effect levels for hatched individuals (Bagshaw et al., 1986; Rafiee et al., 1986). This indicates that effects of chemicals on dormant stages differ among and within species as well as among toxicants, depending on their mode of action.

In our life cycle toxicity experiment we have used dormant eggs from two different populations; one laboratory population (MBT) and one field population (LRV). Control survival of the MBT clones was higher than of the LRV clones, which might be explained by the fact that MBT ephippia have been produced under similar laboratory conditions as used in our experiments (Persoone et al., 2009). Also, MBT clones appeared more sensitive to pesticide exposure than the ones from LRV. Genetic variability in response to stressors has been frequently observed, both among and within populations (Barata et al., 2002; Oda et al., 2006, 2007). Oda et al. (2007), for example, reported a variation in estimated EC_{50} values for fenoxycarb by a factor of four, between seven strains of *D. magna* from different laboratories. Since dormant eggs are produced by sexual reproduction, each individual hatchling represents a different clone and may reveal somewhat different characteristics and tolerances.

Ecological implications

In this study we have shown that pesticides can have negative effects on hatching and development of *D. magna* dormant eggs under conditions where exposure was maximized: the protective envelope was removed and the dormant eggs were exposed to both optimal hatching conditions and pesticides simultaneously. This scenario might be comparable to pesticide applications during spring, resulting in high concentrations of pesticides in small water bodies (via spray drift, runoff or drainage) coinciding with a peak in hatching from the dormant egg bank. The potential impact of the pesticides on the dormant fraction of the community does not only depend on the application time, but also on the exposure route of the respective pesticides. Fenoxycarb is known to dissipate from the water phase quite rapidly and then to bind to the organic phase of the sediment (half-life in water = 3.9 days, half-life in water-sediment systems = 18.8 days; Sullivan, 2000).

This indicates that sediment might be an important exposure route. This was confirmed by Licht et al. (2004) and Jungmann et al. (2009) who reported negative effects of sediment spiked with fenoxycarb on, respectively, mayfly metamorphosis and emergence of chironomids. It is therefore important that future research on dormant stages also focuses on the effects of chemicals under different environmentally realistic scenarios in order to get more insight into the importance of the exposure route (water vs. sediment exposure), timing of exposure and the importance of the ephippial case in protecting dormant eggs from exposure to pollutants.

There are different pathways in which pollution can affect structure and functioning of aquatic communities, or interfere with the life cycle of *D. magna* (Fig. 1). Our observation that even a brief exposure to pesticides during the early stages of embryonic development of dormant eggs leads to reduced hatching rates (fenoxycarb; pathway 2 Fig. 1), a higher proportion of deformed embryos (fenoxycarb; pathway 1+2 Fig. 1) and higher mortality and decreased performance of hatchlings (both pesticides; pathway 3+4 Fig. 1) can have far-reaching consequences for ecological and evolutionary dynamics of zooplankton in lakes and ponds. Reduced hatching rates and performance will reduce population growth rates. A reduced build-up of *Daphnia* early in the season may impact algal growth (reduced top-down control of algae) and fish (less food), and to the extent that the responses are species specific, they may impact species composition of the zooplankton communities. Arbačiauskas and Gasiunaite (1996) and Arbačiauskas and Lampert (2003) showed that ex-ephippial individuals are characterized by different life history traits, fostering faster population growth rates. The impact of pesticides on hatching rates and the fitness of hatchlings thus may even have a higher impact given the importance of the ex-ephippial generation to population development. In addition, repeated pesticide exposure, leading to reduced hatching rates and mortality, may eventually gradually exhaust the dormant egg bank. Since dormant egg banks integrate genetic variation that has accumulated over many growing seasons (Ellner and Hairston, 1994; De Meester et al., 2006), a decrease in size of the dormant egg bank or a reduced contribution of the dormant to the active phase may reduce genetic variation, hence the evolutionary potential of a population (Levin, 1990; Brendonck and De Meester, 2003). This makes exposed populations more vulnerable to changes in environmental conditions of both natural and anthropogenic origin. Despite the importance of dormant egg banks for ecological and evolutionary processes, experimental evidence regarding the influence of toxicants on dormant egg bank dynamics is surprisingly scarce. A better understanding of the effects of toxicant exposure on the full life-cycle of zooplankton species, especially of the important model organism *D. magna*, could increase the ecological relevance of ecotoxicity testing and aid our understanding of not only the impact of pesticide exposure, but also the potential for recovery in aquatic ecosystems.

Acknowledgements

This research was funded by a Ph.D. grant of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen). The authors would like to thank Mieke Jansen for her valuable comments during preparation of the manuscript.

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CHAPTER 3

Timing matters: sensitivity of *Daphnia magna* dormant eggs to fenoxycarb exposure depends on embryonic developmental stage

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Aquatic Toxicology (2015) 159: 176–183

Abstract

Although *Daphnia magna* is a key species in many lentic freshwater ecosystems and is commonly used as model organism in ecology and ecotoxicology, very little is known about the effects of chemicals on their dormant life stages. Dormant eggs (ephippia) are produced when environmental conditions deteriorate, and *Daphnia* switch from clonal to sexual reproduction. Ephippia produced over different growing seasons can accumulate in the sediment of ponds and lakes, where they can be exposed to pesticides and other (anthropogenic) stressors. In the present study, we have investigated the effects of pesticide exposure on dormant eggs at different embryonic developmental stages and evaluated the degree of protection against pollution provided by the ephippial case. We therefore conducted a hatching experiment in which decapsulated and encapsulated dormant eggs were exposed to an insect growth regulator (fenoxycarb) at different stages during their development, both before and after activation of the eggs. In addition, we developed an analytical method to measure fenoxycarb concentrations in the dormant eggs. Fenoxycarb negatively affected development and hatching success and changed the timing of hatching in activated and in dormant eggs. Hatching characteristics as well as fenoxycarb concentrations inside the eggs differed significantly between exposure treatments. Final stages of embryonic development were most sensitive to pesticide exposure and had the highest tissue concentrations of fenoxycarb. Tissue concentrations did not differ significantly between decapsulated and encapsulated eggs, suggesting that the ephippial case offers limited or no direct protection against pesticide exposure. With this study we provide new evidence showing that pesticides can bioconcentrate in and affect *D. magna* dormant eggs. The severity of the effects on developing embryos depends on the timing of pesticide exposure. Our results stress the importance of considering the full life-cycle of model organisms used in ecotoxicological studies, since these are ultimately aimed at assessing risks of chemical exposure on natural aquatic ecosystems.

Introduction

Cyclical parthenogenesis is a mixed reproductive strategy, combining both sexual and asexual reproduction (Bulmer, 1982; De Meester et al., 2004). This strategy is especially common and well-studied in monogonont rotifers, aphids and cladocerans (Decaestecker et al., 2009). Like many other cladocerans, most *Daphnia* reproduce clonally under favorable environmental conditions, but switch to sexual reproduction when conditions deteriorate. Changes in, amongst others, oxygen level, food quantity and quality, photoperiod, temperature and predation can induce the production of males, which in turn fertilize sexual females, leading to the formation of dormant eggs (Alekseev and Lampert, 2001; Slusarczyk et al., 2005; Koch et al., 2009; Fig. 1A). Unlike parthenogenetic eggs, these dormant eggs have thick multi-layered membranes (Seidman and Larsen, 1979; Zaffagnini, 1987; Fig. 1C) and are encapsulated in a protective structure, called ephippium (Schultz, 1977; Ebert, 2005; Fig. 1B), that protects them from mechanical damage and digestive enzymes of organisms like fish and birds (Mellors, 1975; Radzikowski, 2013). Dormant eggs can be dispersed to other water bodies or sink to the sediment layer, where they can remain viable for several decades to centuries (Frisch et al., 2014). Since only a fraction of the dormant eggs hatches each growing season, extensive mixed egg banks are build up over time, containing ephippia produced over different generations, thereby creating a buffering effect in terms of population dynamics and genetic diversity (Caceres, 1997; Brendonck and De Meester, 2003; De Meester et al., 2006).

Daphnia plays a key role in many aquatic systems (Miner et al., 2012). It also is a well-established model organism in ecological and evolutionary research (Lampert and Kinne, 2011) and is used as standard test organism in ecotoxicology (Walker, 2014). Ecotoxicological studies using *D. magna*, performed according to internationally accepted guidelines (OECD TG 202; OECD, 2004; OECD TG 211; OECD, 2012), generally focus on the effects of chemicals on the asexual part of the reproduction cycle, i.e. on clonal lineages consisting of genetically identical females. Despite its ecological importance, considerably less attention has been paid to the effects of chemicals on the sexual part of the reproduction cycle. Endpoints that could be affected by chemical exposure are: offspring sex ratio, dormant egg production and hatching success of dormant eggs. Of these, chemically induced production of male neonates has received increasing attention over the past years (Dodson et al., 1999; Olmstead and LeBlanc, 2003; Tatarazako and Oda, 2007; Palma et al., 2009). This resulted in the inclusion of offspring sex ratio as an additional optional endpoint in OECD TG 211 (OECD, 2012), mainly used to screen chemicals for potential endocrine disrupting effects. So far however, surprisingly little is known about effects of chemicals on the other two endpoints. Olmstead and LeBlanc (2001) have shown that methoprene, a juvenile hormone analog, is able to affect sexual reproduction in *D. magna* (male production was significantly delayed and increased, while dormant egg production was decreased). Also, the surfactant nonylphenol, has been shown to reduce the production of dormant eggs in *D. magna* (Shurin and Dodson, 1997).

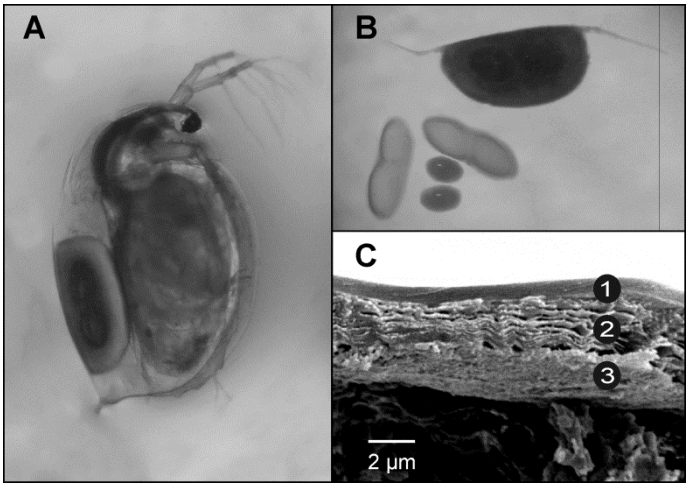

Fig. 1 A) Ephippial *D. magna* female; B) *D. magna* ephippium, opened to show two dormant eggs and the inner envelope, which normally surrounds the dormant eggs; C) membrane structure of *D. magna* dormant egg (scanning electron microscope image): 1 = outer membrane; 2 = middle membrane; 3 = inner membrane.

A few studies have tested the effects of organic chemicals on hatching of *Daphnia* dormant eggs: the fire retardant Fire-Trol® 934 (Angeler et al., 2006), the insecticide fenoxycarb (Navis et al., 2013; Chapter 2) and the biocides menadione and sodium hypochlorite (Raikow et al., 2006; Raikow et al., 2007), have been shown to negatively affect dormant egg hatching success of *D. curvirostris*, *D. magna* and *D. mendotae*, respectively.

In the current study, we aim to expand our understanding of how and to which extent pesticides can affect development and hatching of *D. magna* dormant eggs, by determining the time window during which developing eggs are most sensitive to chemical exposure and by studying the protective value of the ephippial case. For this, we performed a hatching experiment, in which both decapsulated and encapsulated dormant eggs were exposed to the insect growth regulator fenoxycarb at different time periods during embryonic development, corresponding to different developmental stages (Fig. 2). In addition, we developed an analytical method that allows the measurement of fenoxycarb concentrations in the dormant eggs (Flemish Institute for Technological Research VITO NV, Mol, Belgium). To our knowledge, this is the first time that pesticide concentrations have been measured directly in the tissue of *D. magna* dormant eggs. Some earlier studies (Wyn et al., 2007; Chiaia-Hernandez et al., 2013) have measured toxicant levels in dormant stages of *Daphnia,* but using homogenized ephippia, i.e. including the eggs as well as the ephippial case. The concentrations reported could therefore include chemicals retained by the ephippial case and may not necessarily reflect concentrations of toxicants inside the eggs. We used our analytical method to detect whether tissue concentrations differed between treatments that were used in the hatching experiment. Our hypothesis was that the extent to which fenoxycarb penetrated the eggs would be related to effects on development and hatching, and would differ between developmental phases. The eggs were expected to be least sensitive to fenoxycarb exposure when they were still dormant (i.e. before hatching was initiated by light exposure), while later developmental stages were expected to be more sensitive.

Material & Methods

Daphnia magna *dormant eggs*

As starting material for the experiments, ephippia from Langerodevijver, a shallow lake situated in nature reserve “Doode Bemde” close to Leuven (Belgium) were used. Sediment of this lake is known to contain a high density of *D. magna* ephippia (Rousseaux, 2011), with a high hatching success under optimal hatching conditions (Navis et al., 2013; Chapter 2). The top 5-10 centimeters (active egg bank: Caceres, 1998) of the dormant egg bank was sampled in December 2012. In the winter period the egg bank has its maximum size and eggs are in diapause. Pooled sediment samples were sieved (1 mm and 250 µm sieves) and stored for one year at 4°C in the dark before ephippia were manually isolated from the sediment fraction. This storage period ensured that diapause was terminated, the eggs became quiescent and hatching could be induced under favorable conditions (Stross, 1971; Vandekerkhove et al., 2005). For both experiments, isolated ephippia were kept under storage conditions until the start of the experiment, and all manipulations were performed in a room with only red light (700 nm), to prevent unwanted activation of the dormant eggs by light exposure. All eggs were thus dormant prior to incubation under optimal conditions (20°C and a photoperiod of 16h light: 8h dark) and hatching was induced by light activation (47.2 µmoles/m^2/s). For certain treatments, dormant eggs were mechanically decapsulated with metal tweezers shortly before the start of the experiments and only healthy eggs were used.

Fenoxycarb

Fenoxycarb, a juvenile hormone mimicking insecticide, was selected as a model pesticide, because it is known to interfere with hatching success and embryonic development of *D. magna* parthenogenetic (Mu and Leblanc, 2004) and dormant eggs (Navis et al., 2013; Chapter 2). Fenoxycarb (ethyl2-(4-phenoxy-phenoxy)ethylcarbamate, CAS no. 72490-01-8, 99.6% purity, Sigma-Aldrich, Germany) was dissolved in absolute ethanol (purity min. 99.8%, VWR International, France), with a final concentration of 0.05% ethanol in all treatment solutions and in the solvent control. Test solutions were freshly prepared on each exposure day and stock solutions were stored at -20°C. Final nominal concentrations of 1 and 4 mg/L fenoxycarb were used. They were chosen based on previous hatching experiments (Navis et al., 2013; Chapter 2) where these concentrations were found to delay and suppress hatching and increase the percentage of malformations in developing embryos. This enabled us to compare the sensitivity of the different embryonic developmental stages to fenoxycarb exposure. The actual concentration of the highest nominal test concentration (4 mg/L) was verified using UPLC-MS/MS and corresponded to a measured concentration of 3.24 mg/L (average of four samples).

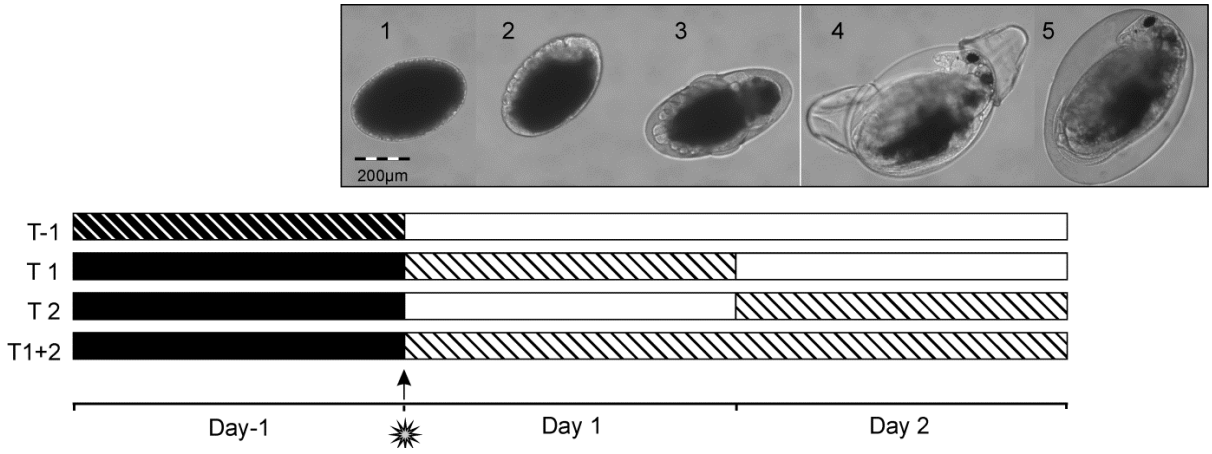

Fig. 2 Stages of normal embryonic development of *D. magna* dormant eggs, as observed at 20°C and 16h light: 8h dark (photographs numbered 1-5, similar to developmental stages in parthenogenetic eggs as described by Kast-Hutcheson, 2001). Stage 1: no evidence of cellular differentiation (before and 0-12h after light activation). Stage 2: cellular organization and differentiation, 1st embryonic membrane is shed shortly after entry into stage 2 (12-20h after light activation). Stage 3: embryonic development, pigmented eye is evident, 2nd embryonic membrane starts to rupture (21-30h after activation). Stage 4: embryonic maturation, head capsule and second antennae have differentiated. Ruptured 2nd embryonic membrane is often visible as caps on inner membrane (31-45h after activation). Stage 5: Late embryonic maturation, antennae partially extended, shell spine folded against carapax. Volume inside the inner membrane increases until rupture of membrane (minimum 48 hours after activation). After that the organism is hatched and free swimming, second antennae setae are developed and shell spine is fully extended from the carapax. Depicted below the photographs are the four different experimental time-windows of fenoxycarb exposure (indicated as the hatched part of the bars), before and after light activation of the eggs (black is before activation, white after): T-1, 1 day exposure in the dark (dormant eggs); T1 exposure during the 1st day after light activation; T2 during the 2nd day; T1+2 during both days.

Hatching experiment

In a first experiment, both decapsulated (D) and encapsulated (ephippia; E) eggs (Fig. 1B) were exposed to fenoxycarb at different stages during embryonic development. Their hatching characteristics and the occurrence of developmental malformations were subsequently monitored during 10 days. Eggs were exposed to fenoxycarb during one of four exposure times: from 24 h before light activation until just before light activation (T-1); and at different time periods after light activation: only during the first 24 h (T1); from 24 h until 48 h (T2); or for 48 h, starting directly after light activation (T1+2). These exposure times coincide with different developmental stages during the hatching process of dormant eggs (Fig. 2) as observed in previous experiments (Navis et al., 2013; Chapter 2): T1 corresponds to early embryonic development and T2 to late embryonic development. The dormant (T-1) or activated (T1, T2 and T1+2) eggs were exposed to either 1 or 4 mg/L fenoxycarb or to control conditions. This resulted in a total of 26 experimental treatments; 4 exposure times (T-1, T1, T2 and T1+2) * 3 pesticide concentrations (0, 1 or 4 mg/L fenoxycarb) + 1 solvent control for T1+2 (the longest exposure time) = 13 exposure treatments * 2 egg types (D and E). For each treatment, 48 decapsulated eggs (D) or 48 ephippia (E) were placed individually in the wells of 24-well microtiter plates (polystyrene, non-coated, sterile plates, Greiner Bio-One GmbH) containing 2 mL of exposure medium, prepared in artificial freshwater (ADaM: Klüttgen et al., 1994).

To ensure that exposure to the pesticide could only occur during the pre-determined time window, the eggs were rinsed and transferred into new microtiter plates with 2 mL fresh ADaM-water after the exposure period had ended. Treatments were randomized over plates in such a way that each treatment was allocated to four randomly assigned half multiwell plates. For treatments T1, T2 and T1+2, plates were incubated for 10 days at 20±2°C in a light:dark regime of 16:8h, to simulate spring conditions and induce hatching. Under these specific conditions, hatching of eggs from this population is known to be very successful (above 80%) and synchronized (Navis et al., 2013; Chapter 2). For treatment T-1, plates were first exposed to fenoxycarb in total darkness at 20±2°C, simulating conditions preceding the activation of dormant eggs, and transferred to light conditions after the 24 hours of dark exposure had ended. All incubated eggs were checked daily for hatching. At the end of the experiment (day 10), all ephippia (E) were opened and checked for any remaining (unhatched) eggs. In addition, embryonic development and morphological abnormalities were monitored using a stereomicroscope (for decapsulated eggs daily during the hatching experiment, for eggs encased in ephippia only after termination of the experiment).

Fenoxycarb in dormant egg tissue – experiment and analytical method

In a second experiment, tissue concentrations of fenoxycarb in the developing embryos were analysed. For this, dormant eggs of *D. magna* (either decapsulated or encapsulated eggs) were exposed to 4 mg/L fenoxycarb at similar exposure treatments as in the previous hatching experiment. This resulted in 8 experimental treatments; 4 exposure times (T-1, T1, T2 and T1+2) * 1 concentration fenoxycarb (4 mg/L) * 2 egg types (D and E). For each treatment three replicates were included, and per replicate 100 eggs (D) or 100 ephippia (E) were exposed in the wells of a 6-well microtiter plate with 6 mL of exposure solution. Dormant eggs were removed from the ephippia directly after the exposure period had ended and only the eggs were used for further analysis to exclude fenoxycarb retained by the ephippial case. All eggs were rinsed in fresh ADaM-water, collected into cryotubes (100 eggs per tube) and stored at -80°C until further analysis.

To a sample consisting of 100 *Daphnia* eggs, 1 mL of pure methanol was added and the mixture was vortexed during 1 min. After 1 h of equilibration, the mixture was vortexed again and subsequently centrifuged at 16000 g (Galaxy 16DH ultracentrifuge, VWR, Belgium). The supernatant was 1/1 diluted with water and 10 µL of this solution was injected into the UPLC-MS/MS system (Waters Acquity UPLC system coupled to a Waters Xevo-TQS triple quadrupole mass spectrometer, with a Waters Acquity BEH C18 (1.7 µm, 100*2.1 mm) column at 40°C). The mobile phase consisted of a water/acetonitrile/4 mM ammonium acetate/0.1% formic acid gradient. The mass spectrometer was operated in positive electrospray mode and fenoxycarb was detected on the basis of the MRM transition 301.98 (precursor ion)>87.8 (product ion). The cone voltage was set at 40V, the collision energy at 19V. The peak of fenoxycarb was integrated and the concentration was calculated using external calibration. A linear correlation between peak area and concentration was obtained, with a correlation coefficient (r^2) of >0.995. The average recovery was 86%; the recovery was determined for eggs fortified at a concentration of 40 ng/100 eggs.

The limit of quantification, which was calculated as the concentration corresponding to 6 times the chromatogram noise of a 0.3 µg/L extract, was 0.02 ng/100 eggs (or 0.02 µg/L in the extract). All reagents used were analytical grade and obtained from commercial sources; methanol and acetonitrile (UHPLC–grade, Fisher, Belgium), formic acid (>98%, Merck), fenoxycarb and ammonium acetate (resp. purity 99.5% and 99.9%, Sigma-Aldrich, Belgium). Water used was purified using a Milli-Q Direct-Q3 system (Millipore, Milford, MA, USA). Individual standard solutions of fenoxycarb were prepared in methanol at concentrations of 0.09 to 34 µg/L and stored at 4°C.

Statistical analysis

For the hatching experiment, both hatching success as well as developmental malformations of the embryos (in %) were related to timing and concentration of fenoxycarb exposure as well as to egg type (decapsulated and encapsulated) using generalized linear models (GLM) with a logit-link function and binomial distribution, followed by sequential Bonferroni-correction (Holm, 1979), to correct for multiple testing. Plate identity was taken into account by including it as a random blocking factor. In the hatching experiment, blank controls were included for each exposure time while a solvent control treatment was incorporated for the longest fenoxycarb exposure (T1+2). Effects of the pesticide on timing of hatching (day of maximum hatching) were evaluated only for decapsulated eggs, using two-way ANOVA´s followed by Tukey´s HSD post-hoc tests. For encapsulated eggs, this was not possible, since hatched (but deformed) individuals still present in the ephippia were only observed upon opening of the ephippia after termination of the experiment, making it impossible to determine their actual day of hatching. Tissue concentrations of fenoxycarb were log transformed, in order to ensure homogeneity of variances (Levene´s test) and the effect of exposure time and egg type on the concentrations in the eggs were also analysed using two-way ANOVA´s and Tukey´s HSD post-hoc tests. All statistical analysis were performed in R statistical software v3.0.2 (The R Foundation for Statistical Computing, 2013).

Results

Hatching experiment

Both fenoxycarb exposure and egg type (decapsulated vs encapsulated) had a significant effect on hatching success of *D. magna* dormant eggs (Table 1). The extent of the effects differed between the four exposure times. When exposure took place during the two days after activation (i.e. incubation under light conditions) (T1+2), or only on the second day after activation (T2), hatching success of the decapsulated eggs was significantly negatively impacted by fenoxycarb (reduction of 26.6% and 33.5% at 4 mg/L fenoxycarb for T1+2 and T2, respectively; Fig. 3C+D). For the other two exposure times no significant effect on hatching of decapsulated eggs was observed. Hatching of encapsulated eggs was significantly negatively impacted by fenoxycarb exposure at T-1 (1 day in the dark) and T1+2 (2 days, in light); hatching in these treatments was reduced with 37.4% and 39.7%, respectively (Fig. 3A+D).

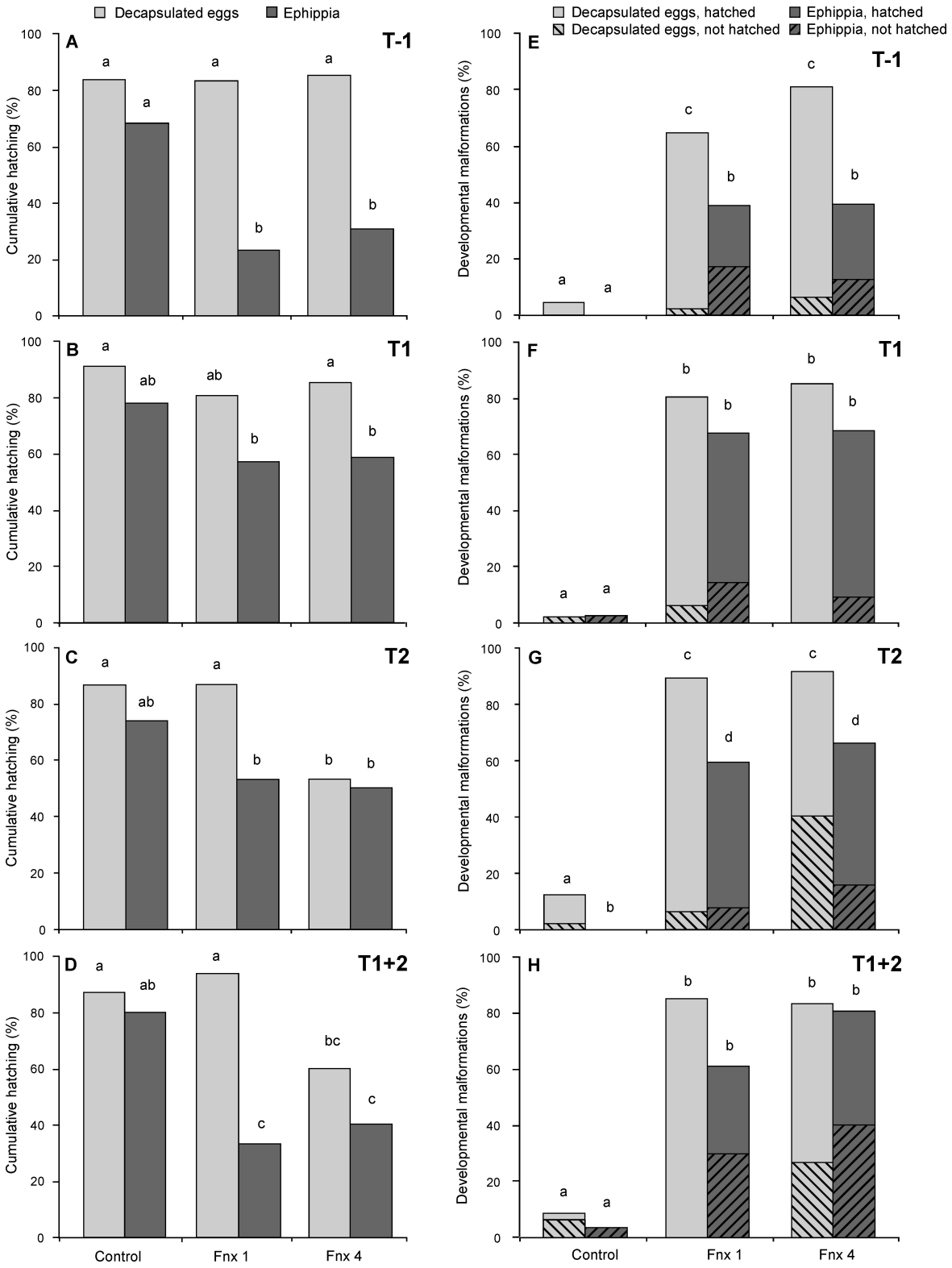

Fig. 3 Effects of fenoxycarb (fnx) treatment at four different exposure times (T-1, T1, T2 and T1+2) on cumulative hatching percentage during 10 days after exposure (left panel) and on the percentage of deformed hatchlings (right panel). T-1 = dark exposure for 1 day, T1 = exposure on day 1, T2 = exposure on day 2, T1+2 = exposure on day 1 and 2. Fnx1 = fenoxycarb 1 mg/L, Fnx4 = fenoxycarb 4 mg/L, nominal test concentrations. Light grey bars represent decapsulated eggs (D) and dark grey bars eggs encapsulated in their ephippial case (E) during exposure. Hatched parts of the bars indicate deformed embryos that did not hatch. Distinct letters in the figures indicate significant differences among treatments within exposure times (n = 48, $p < 0.05$, generalized linear model, followed by sequential Bonferroni-correction).

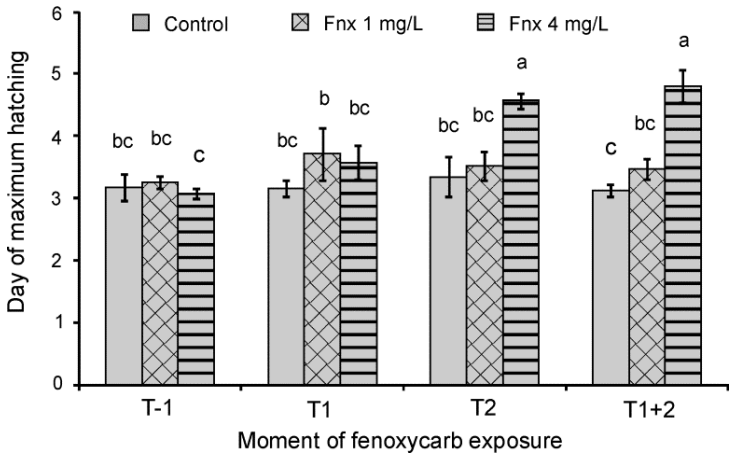

Fig. 4 Effects of fenoxycarb (Fnx) exposure on day of maximum hatching, for the four different exposure times (T-1, T1, T2, T1+2) and for decapsulated eggs only. At T2 and T1+2 hatching was significantly delayed at the highest fenoxycarb concentration (4 mg/L). Distinct letters in the figure indicate significant differences (n=12, $p < 0.05$, 2-way ANOVA, followed by Tukey´s HSD post-hoc test).

Even in treatments where fenoxycarb had no direct effects on hatching, it caused malformations in developing embryos (deformities of tail spine, antennae, carapax and compound eye were observed). This increase in developmental malformations was significant (Table 1) at all four exposure times and both fenoxycarb concentrations (Fig. 3E-H). Malformations in embryos from decapsulated eggs were most severe when exposed at T2 and T1+2. At these two exposure times hatching was also significantly delayed (Fig. 4). The percentage of developmental malformations was lowest in encapsulated eggs exposed at T-1; many eggs (55-59%) from this treatment did not develop at all and remained in stage 1. Most deformed embryos exposed at T-1 and T1 could still hatch, but at T2 and T1+2 there was a large fraction of deformed embryos that were unable to hatch (40.4% and 27.1% resp. for decapsulated eggs at 4 mg/L fenoxycarb).

Table 1 Results of generalized linear model of effects of fenoxycarb exposure (fnx conc), window of exposure (time), egg type and their interactions, on cumulative hatching after 10 days and developmental malformations in hatched individuals.

	Chi^2	Df	P-value
Cumulative hatching (%)			
Fnx conc	98.43	3	< 0.001 *
Time	21.61	3	< 0.001 *
Egg type	118.57	1	< 0.001 *
Fnx conc * Time	10.08	6	0.121
Fnx conc * Egg type	22.93	3	< 0.001 *
Time * Egg type	12.97	3	0.005 *
Fnx conc * Time * Egg type	16.85	6	0.010 *
Developmental malformations (%)			
Fnx conc	711.50	3	< 0.001 *
Time	45.43	3	< 0.001 *
Egg type	72.01	1	< 0.001 *
Fnx conc * Time	2.91	6	0.820
Fnx conc * Egg type	4.20	3	0.241
Time * Egg type	7.18	3	0.066
Fnx conc * Time * Egg type	7.48	6	0.278

Fenoxycarb concentration in eggs

Concentrations measured in the eggs ranged from 15.7 to 1337.3 ng/100 eggs (Fig. 5). The concentrations of fenoxycarb differed significantly among the exposure times ($F = 89.664$; $df = 3$; $p < 0.001$), but not between the two egg types ($F = 0.824$; $df = 1$; $p = 0.377$). Lowest concentrations were detected in dormant eggs that were encapsulated in their ephippium during exposure to fenoxycarb for 1 day while still in the dark (T-1). Highest concentrations were detected in decapsulated eggs, exposed during the 2nd day after activation by light (T2) (Fig. 5). Average concentrations (mean of three replicates) for these treatments were; 19.6 ng/100 eggs for encapsulated eggs from T-1 and 923.2 ng/100 eggs for decapsulated eggs from T2.

In Figure 6, we integrated the results of hatching success and intra egg pesticide concentrations. For decapsulated eggs, treatments with the highest tissue concentration of fenoxycarb coincided with lowest hatching success (Fig. 6A). Even at low tissue concentrations, fenoxycarb caused developmental malformations in the embryos (Fig. 6B). And in all treatments a very large portion (>80%) of the hatchlings was deformed (Fig. 6C).

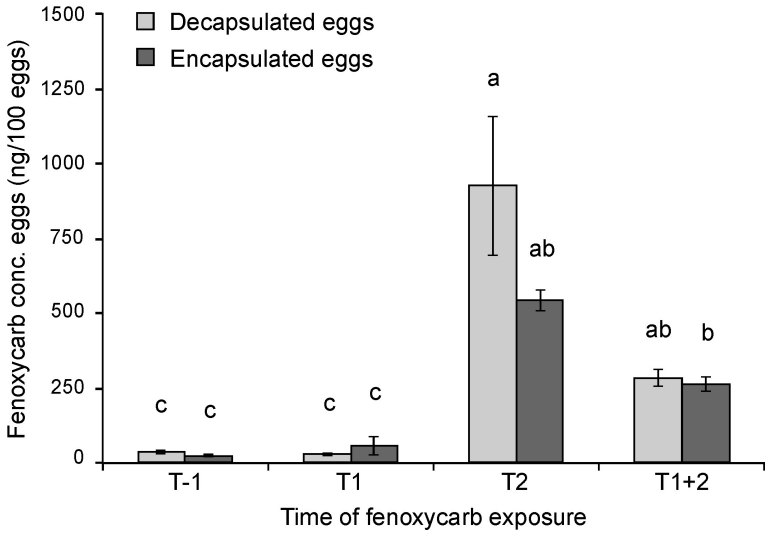

Fig. 5 Concentrations of fenoxycarb measured by UPLC-MS/MS in *D. magna* dormant eggs; light grey bars indicate decapsulated eggs, dark grey bars represent eggs encapsulated in their ephippial case during exposure. The two egg types were exposed to 4 mg/L fenoxycarb at four different stages during embryonic development (T-1, T1, T2 and T1+2). Highest fenoxycarb concentrations were detected in embryos exposed during later developmental stages (T2 and T1+2). Distinct letters in the figure indicate significant differences ($p < 0.05$, 2-way ANOVA, followed by Tukey´s HSD post-hoc test).

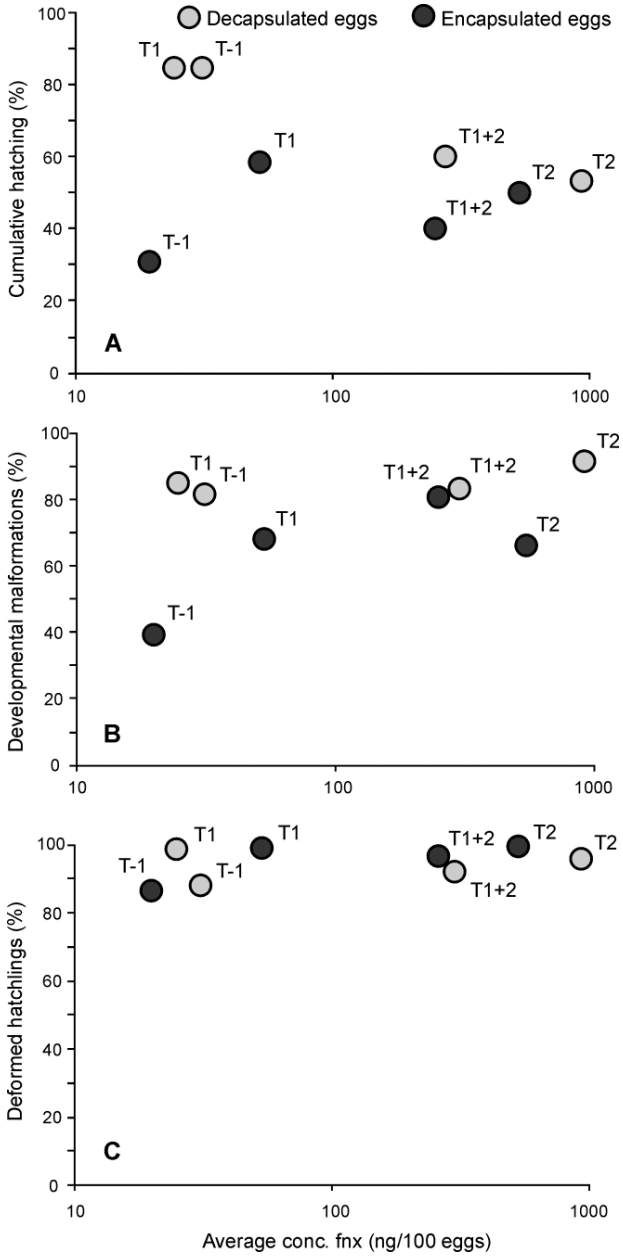

Fig. 6 [1] Correlation between concentrations of fenoxycarb measured in egg tissue (average fenoxycarb concentration of three replicate samples, expressed in ng/100 eggs) and A) cumulative hatching success (%), B) developmental malformations (%) observed in the embryos, and C) deformed hatchlings (%). Light grey circles represent treatments with decapsulated eggs, dark grey circles with encapsulated eggs, exposure times (T-1, T1, T2 and T1+2) are indicated next to the corresponding symbols.

[1] Figure 6 depicts a graphical representation of the measured internal egg concentrations and three endpoints related to the hatching experiment, intended to integrate and visualize results of the different experiments. The relationship between these variables has not been modeled or tested statistically.

Discussion

Although *D. magna* is a key species in many freshwater ecosystems (Lampert and Kinne, 2011; Miner et al., 2012) and one of the most commonly used test species in ecotoxicology (Walker, 2014), very little is known about the effects of chemicals on their dormant life stages. In previous work we have demonstrated that pesticides can not only affect development and hatching success of *D. magna* dormant eggs, but also survival and performance of the individuals hatched from exposed eggs (Navis et al., 2013; Chapter 2). In the present study, building further on these findings, we have shown that, a) the time-window of exposure during embryonic development determines the impact on hatching success and timing as well as the severity of deformations in embryos and hatchlings, and b) these effects can be related to the internal fenoxycarb concentrations as measured in the egg tissue. In addition, fenoxycarb tissue concentrations in decapsulated versus encapsulated eggs did not differ significantly, suggesting that the ephippial case offers limited or no direct protection against fenoxycarb exposure.

Timing of exposure determines impact of fenoxycarb on developing embryos

As hypothesized, the negative effects of fenoxycarb on embryonic development and hatching characteristics (cumulative percentage and timing) of *D. magna* dormant eggs, differed significantly between the four exposure times. The final stages of embryonic development (Fig. 2 Stages 4 and 5) were most sensitive to fenoxycarb exposure. Before light activation, dormant eggs are surrounded by three membranes (Zaffagnini, 1987), after activation and during embryonic development these membranes are shed and at the last developmental stage, embryos are only protected by one external membrane and become much more active. This allows a higher influx of the surrounding medium (Davison, 1969) and thus also fenoxycarb into the eggs. Bodar et al. (1989) similarly found that the last embryonic instars of developing parthenogenetic eggs of *D. magna* were most sensitive to metal exposure.

Bioconcentration of fenoxycarb in dormant egg tissue

Previously, we have shown that the extent to which pesticides can affect dormant eggs of *D. magna* can differ between pesticides (Navis et al., 2013; Chapter 2): fenoxycarb had significant negative effects on development and hatching, while there were no such direct effects of carbaryl. We suggested that this could be related to the toxicants mode of action and difference in the ability of the two pesticides to bioconcentrate in the eggs. In this study we have proven that fenoxycarb is indeed taken up from the water fraction by dormant eggs of *D. magna* and that the level of bioconcentration depends on the timing of pesticide exposure during embryonic development. Fenoxycarb levels in eggs exposed before light activation (T-1) or during the first developmental stages (T1) were relatively

Our findings on bioconcentration of fenoxycarb cannot directly be compared to other studies, since, to our best knowledge, this is the first study to quantify pesticide concentrations inside *Daphnia* dormant eggs. In two previous studies, metals (Wyn et al., 2007) and several organic contaminants (Chiaia-Hernandez et al., 2013) were measured in whole *Daphnia* ephippia (including the eggs as well as the protective maternal case) while in a diapausing state. Both studies found that contaminants were taken up in ephippia. Bioconcentration of organic compounds depended mainly on their hydrophobicity. For diazinon, a pesticide with a similar log K_{ow} as fenoxycarb (but a different mode of action), a bioconcentration factor of 170 L/kg_{lip} has been reported (Chiaia-Hernandez et al., 2013). However, a substantial number of ephippia in this study did not contain any eggs (around 80%). Therefore it is likely that part of the toxicant levels measured, actually consisted of chemicals retained/absorbed by the ephippial case, instead of the egg tissue.

Protective value of ephippial case

One of our aims was to identify whether the ephippial envelope would protect the dormant eggs against toxicant exposure. Tissue concentrations did not differ significantly between decapsulated versus encapsulated eggs at any of the four exposure windows. This observation suggests limited or no direct protection of the ephippial case against chemical exposure of the embryos. Instead, the effect of the pesticide exposure seems to be determined by the (number of) membranes surrounding the embryo during the exposure period. In addition, fenoxycarb had an even stronger effect on hatching success of encapsulated than decapsulated eggs (Fig. 3A-D). A possible explanation for this might be that embryos from encapsulated eggs needed more energy to hatch (extra cost of getting out of the ephippial case), leading to a stronger negative effect of pesticide exposure.

Ecological implications

With this study we contribute to existing evidence showing that pollutants cannot only affect survival and reproduction of clonal lineages of *Daphnia*, but also have effects on hatching of dormant eggs (Angeler et al., 2006; Raikow et al., 2006; Raikow et al., 2007; Alekseev et al., 2010; Navis et al., 2013; Chapter 2). Even though dormant eggs show a high tolerance to extreme physical conditions like freezing and desiccation (Mellors, 1975; Radzikowski, 2013), they can still be affected by chemical pollution. Effect levels of fenoxycarb for development and hatching are about twice the acute effect levels for neonates: 48h EC_{50} of *D. magna* neonates is 0,5-0,6 mg/L (EFSA, 2010), while the EC_{50} for hatching is 1,3 mg/L (Navis et al., 2013; Chapter 2). Embryonic development of parthenogenetic eggs seems to be affected at concentrations about 1000 times lower (Mu and LeBlanc, 2004) than embryonic development in dormant eggs. This indicates that, with respect to fenoxycarb exposure, dormant eggs are less sensitive than endpoints related to the asexual part of the reproduction cycle in *D. magna*. Our study clearly indicates that the severity of the effects of pesticide exposure on developing dormant eggs of *D. magna* depends on the timing of exposure, with the later developmental stages being most sensitive. However, eggs were not only affected during embryonic development (after activation by light incubation), but even when they were still dormant (in dark conditions) and when surrounded by an ephippial case.

Depending on the reversibility of these effects, exposure to certain types of chemicals could therefore impact the size and structure of zooplankton dormant egg banks, in turn affecting the benthic-pelagic coupling in aquatic systems (Gyllström and Hansson, 2004; Angeler and Garcia, 2005). Interference with development and hence a reduced hatching from the mixed egg bank may have effects on (re)colonization of aquatic systems. Small active population sizes could further lower the amount of produced dormant stages, eroding the buffering capacity of the egg bank against the risk of local extinction and loss of genetic diversity (Levin, 1990; Brendonck and De Meester, 2003). In current higher-tier studies concerning potential long-term impacts of chemical exposure on zooplankton communities and populations, impacts on dormant life stages are generally not taken into account (e.g. Beketov et al., 2008; Stampfli et al., 2011). This could result in incomplete or false assessments regarding the potential for recovery of aquatic ecosystems that were previously exposed to pesticides. Our results stress the importance of considering the full life-cycle of model organisms used in ecotoxicological studies, that are ultimately aimed at assessing risks of chemical exposure on natural aquatic ecosystems.

Acknowledgements

This research was funded by a Ph.D. grant of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen) and the KU Leuven Research Fund (Excellence Center Financing PF/2010/07). The authors would like to thank Camille De Raedemaeker and Ruben Cardoen for their practical assistance during the laboratory experiments.

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CHAPTER 4

Acute and chronic effects of exposure to the juvenile hormone analogue fenoxycarb during sexual reproduction in *Daphnia magna*

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Submitted manuscript

Abstract

In the last two decades, several studies have demonstrated that insect growth regulating insecticides are able to affect reproduction in a number of zooplankton species at very low exposure levels. In the cyclic parthenogenetic water flea *Daphnia*, most of this research has focused on the asexual part of the life cycle and on the induction of male offspring. Much less is known about effects on the quantity and quality of sexual, dormant eggs. Using fenoxycarb as a model pesticide, we exposed male and female neonates, under conditions inducing a switch to sexual reproduction, and tested for effects on dormant egg (ephippia) production and sex ratio of parthenogenetic offspring. We then also assessed whether fenoxycarb exposure affected the viability of the produced dormant eggs, as well as survival and life-history characteristics of hatched individuals. Our results show that exposure to fenoxycarb concentrations of 1 µg/L or higher caused a decrease in both parthenogenetic and sexual egg production, while inducing the production of male offspring. Tested fenoxycarb concentrations are in the range of peak levels found in the environment after pulse exposures. There were no significant effects of fenoxycarb exposure on the survival and life history characteristics of the hatchlings. This indicates that even though the quantity of dormant eggs was reduced, the quality of the eggs was not affected significantly by fenoxycarb exposure. Impacts on both the sexual and asexual reproductive phase in *D. magna*, through changes in quantity and timing of male versus female offspring and ephippia production, could affect the active aquatic phase as well as the dormant phase in zooplankton populations.

Introduction

Over the past few decades, considerable attention has been paid to (potential) endocrine disruption in wildlife caused by chemical substances, so called endocrine disruptors. A widely used definition for an endocrine disrupting chemical (EDC) is “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations” (WHO, 2002). The most well-known examples are probably organo-chlorine pesticides (mainly DDT) causing eggshell thinning in predatory birds (Grasman et al., 1998), and the anti-fouling agent tributyltin causing imposex in marine snails (Waite et al., 1991; Evans and Nicholson, 2000). Many more studies have identified chemicals that exhibit hormone-like activity (reviewed in Sumpter, 2005; Rodriguez et al., 2007; Tatarazako and Oda, 2007). Valid endpoints and experimental setups to test for endocrine disruption remain heavily debated and to date no clear cut testing strategies exist (Dietrich et al., 2013; OECD GD 150; OECD, 2012).

Several studies have demonstrated that certain chemicals (mainly insect growth regulating insecticides, IGR´s) are able to mimic methyl farnesoate (MF), an important terpenoid hormone in crustaceans that is involved in the regulation of embryonic development, growth and reproduction, comparable to juvenile hormones in insects (reviewed in LeBlanc, 2007). Exposure to juvenile hormone mimicking substances has been shown to influence asexual reproduction in zooplankton species at very low concentrations, and induces the production of male offspring (Olmstead and LeBlanc, 2001a; Abe et al., 2015). In many so-called cyclic parthenogenetic zooplankton species, including the commonly used model organism *Daphnia magna*, asexual reproduction is alternated with a sexual reproductive phase (Decaestecker et al., 2009). Under favorable conditions *Daphnia* produce asexually (clonal lineages), allowing a rapid population growth during the growing season. When conditions become less favourable (indicated by e.g. low temperatures, crowding, changes in photoperiod, food quantity and quality), the organisms switch to sexual reproduction, where males are produced that fertilize haploid eggs produced by sexual females (Alekseev and Lampert, 2001; Abrusán et al., 2007; Walsh, 2013). The resulting dormant eggs (encapsulated in a protective envelope called ephippium) usually sink to the bottom of the water body and accumulate in so-called dormant egg banks, from which each growing season a fraction can hatch. Unhatched eggs can remain dormant, but viable, for several decades to centuries (Frisch et al., 2014). Delayed hatching from the egg bank is generally considered a risk spreading strategy to cope with environmentally harsh and sometimes unpredictable conditions and appears crucial for the long-term persistence of zooplankton populations (Brendonck and De Meester, 2003). To date, most studies on the impact of toxicant exposure in cyclic parthenogens focused on the asexual reproduction cycle, while consequences for the sexual reproductive phase remain understudied. Stages of the sexual reproductive phase that could be impacted by toxicant exposure are: 1) the switch from parthenogenetic to sexual reproduction, as signaled by the induction of males (offspring sex ratio), 2) the timing and quantity of ephippia production, and 3) the quality of produced dormant eggs (e.g. impacts on hatching success, or survival and life history characteristics of hatched individuals).

Several studies have established that IGR´s can impact sex determination in various zooplankton species (Oda et al., 2005), including *D. magna* (Olmstead and LeBlanc, 2000, 2003; Tatarazako, 2003; Wang et al., 2005). Offspring sex in *Daphnia* is normally determined by environmental conditions, and occurs during later stages of ovarian oocyte maturation (Olmstead and Leblanc, 2002). Exposure to IGR´s can induce the production of male offspring in *Daphnia* clones, producing female broods under control conditions (Oda et al., 2005). To screen for potential endocrine disrupting effects of chemicals, sex ratio has recently been included as an additional, optional endpoint in the *Daphnia* reproduction test (Tatarazako and Oda, 2007; OECD Guideline 211; OECD, 2012). So far, there is little experimental data available regarding effects of toxicants on the various endpoints of the sexual reproduction cycle in *Daphnia.* A few studies have tested for the impact of chemicals on dormant egg production (Shurin and Dodson, 1997; Olmstead and LeBlanc, 2001b; Saika et al., 2006) and they all observed a decrease in ephippia production with increasing toxicant concentration. However, none of these studies tested for effects of toxicant exposure on the quality of the produced dormant eggs, as indicated by hatching success and the survival and life history characteristics of hatched individuals.

In previous studies we determined that fenoxycarb is able to affect embryonic development of *D. magna* dormant and parthenogenetic eggs (Navis et al., 2013, 2015; Chapter 1-3). Fenoxycarb was the only model pesticide we screened that affected not only embryonic development, but also hatching success of dormant eggs (Chapter 1). With this study we aim to increase our understanding of the influence of exposure to the IGR fenoxycarb on both the number and quality of dormant egg production in *D. magna*. We therefore exposed male and female neonates, under conditions inducing a switch to sexual reproduction, and tested for effects on both sexual and parthenogenetic reproduction (Fig. 1, number 1A+1B). Next, after a subsequent cold period, we used the produced dormant eggs in a hatching experiment, to assess their viability (Fig. 1, number 2+3). In addition, survival and life-history characteristics of hatched individuals were also monitored (Fig. 1, number 4).

Material and methods

Daphnia magna – *study population*

D. magna clones originating from a field population “Langerodevijver” (LRV: Korbeek-Dijle, Belgium) were used for all experiments. This population was selected because it is located in a rather pristine environment (nature reserve “Doode Bemde”; Orsini et al., 2012) and contains a high density of *D. magna* (Navis et al., 2013; Chapter 2). Clones used in our experiments were either hatched from ephippia that were isolated from the sediment (top 5 centimeters, active egg bank; Caceres, 1998) or collected directly from the aquatic phase in the winter period of 2011/2012 or 2012/2013. Clones were cultured in the laboratory for a minimum of one year at 20 ± 2°C, a light:dark regime of 16:8h, with the algae *Scenedesmus obliquus* as food, before use in any further experiments.

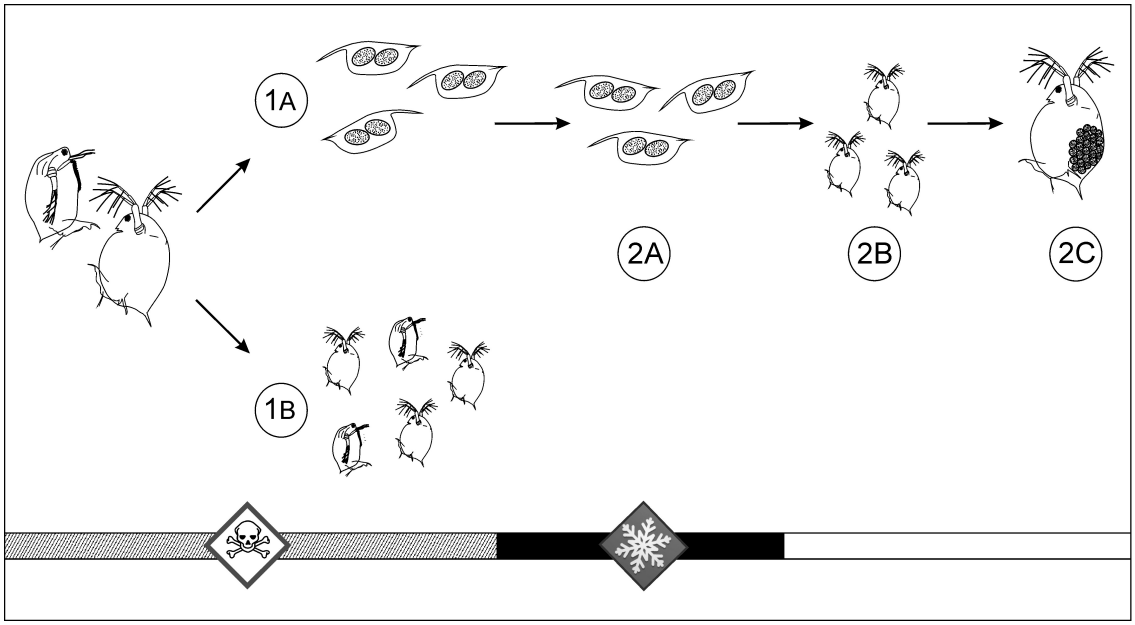

Fig. 1 Effects of fenoxycarb exposure during the sexual reproductive phase in *D. magna*: 1A) on the quantity of dormant eggs produced (number of ephippia); 1B) on the number and sex ratio of parthenogenetic eggs); 2) on the quality of the produced dormant eggs, quantified by 2A) filling percentage and egg volume, 2B) hatching success, and 2C) life history characteristics of hatched individuals (age at maturity, size first brood). Fenoxycarb exposure in this study only took place during the sexual reproductive phase (grey striped bar). Produced ephippia were removed daily from the exposure medium, rinsed and stored in clean medium for two months in cold and dark conditions (black bar), prior to inducing hatching under optimal conditions (white bar).

Fenoxycarb exposure under conditions inducing sexual reproduction

We tested the effects of fenoxycarb exposure under conditions inducing sexual reproduction. For this we added three female neonates (less than 48h old) from each of three different clones (9 females in total) with a high affinity for production of ephippia (selected based on a pilot experiment) to a beaker with 200 mL artificial freshwater (ADaM-medium: Klüttgen et al., 1994). Next, four male neonates produced by a fourth clone were added to the same test vessel. This resulted in a total of 13 individuals in 200 mL, which simulates crowding. To further stimulate sexual reproduction, these neonates were kept under a short-day photoperiod (8h light:16h dark) (De Meester and De Jager, 1993; Alekseev and Lampert, 2001). In addition, the food source was changed to *Nannochloropsis limnetica* ($1*10^5$ cells/ml), because good food quality (mainly fatty acid composition) has been demonstrated to be an important factor in the induction of dormant egg production (Abrusán et al., 2007). After one week, just before the females became mature, they were exposed to six fenoxycarb treatments : 0.1, 1, 10 and 100 µg/L, a blank and a solvent control (0.05% ethanol). We verified that all used test concentrations are sub-lethal in a pilot experiment (Navis, unpublished results; published 48h EC_{50} for *D. magna* neonates = 500-600 µg/L; EFSA, 2010). Each treatment was replicated nine times, which resulted in 6 treatments * 9 replicates = 54 experimental units. For a period of 14 days, the test organisms were exposed to fenoxycarb, and medium was changed every other day. Parthenogenetic offspring and ephippia were removed from the test beakers daily. Collected ephippia were rinsed twice in clean ADaM-medium and transferred into cryotubes covered with aluminium foil and stored at 4°C in the dark.

In addition, mortality of the adults and sex ratio of parthenogenetic offspring was recorded daily. Offspring sex was determined by visual inspection of the first antennae, using a stereomicroscope (Olmstead and LeBlanc, 2000).

After a storage period of eight weeks at 4°C (needed to break diapause and allow the eggs to become quiescent; Stross, 1971; Vandekerkhove et al., 2005), we tested the viability of the produced dormant eggs in a hatching experiment. The collected ephippia were first decapsulated mechanically to determine the filling percentage (100% indicates two eggs were present in the ephippium) and we measured the size of the dormant eggs (longest and shortest axis) to estimate egg volume (Olympus CKX41 inverted microscope, magnification 10x). Dormant eggs were placed individually in the wells of a 24-well microtiter plate (polystyrene, non-coated, sterile plates, Greiner Bio-One GmbH) containing 2 mL ADaM-medium and incubated at 20±2°C and 16h light:8h dark. Eggs originating from different fenoxycarb treatments (two controls, four fenoxycarb concentrations) and collected at different days during the experiment were randomly allocated to plates. Hatching characteristics and development were monitored daily for 10 days.

Finally, to test if survival and important life history variables of the hatched individuals were affected by fenoxycarb exposure of the mothers, we monitored 20 hatchlings from each of the six treatments for their life history traits in a life-table experiment up to production of their first brood. From each treatment, 20 hatchlings were collected from the day of maximum hatching (and subsequent days in case not enough hatchlings were obtained on one day; in most cases the day of maximal hatching was day 3) and transferred individually into 100 mL beakers with ADaM-medium. Survival was monitored daily, and in addition age at maturity and size of the first brood were determined.

Statistical analysis

The effect of fenoxycarb exposure was evaluated using one-way ANOVA´s followed by Tukey´s HSD post-hoc tests, for the following endpoints: number of parthenogenetic offspring, number of ephippia, age at maturity of the hatchlings and size of their first brood. All results regarding reproductive output were corrected for adult mortality and are expressed per surviving female. Effects on offspring sex ratio, adult survival, filling percentage of the ephippia, hatching of the dormant eggs and survival of the hatchlings were statistically evaluated using generalized linear models (GLM) with a logit-link function and binomial distribution, followed by sequential Bonferroni-correction (Holm, 1979). All statistical analysis were performed in R statistical software v3.0.2 (R Development Core Team, 2013). In all experiments a blank control and a solvent control treatment were included. If the results of both control treatments were not statistically different, only results from the blank controls were reported.

Results

Effects on number and sex ratio of juveniles

Survival of the adults (both males and females) in control treatments ranged from 76.9-100%. Adult survival in the fenoxycarb treatments was not significantly different from the control treatments (Table 1). In the control treatments on average (± st.error) 8.2±0.4 juveniles were produced per surviving female over the course of the experiment. In fenoxycarb exposed treatments, parthenogenetic reproductive output was significantly lower (Table 1), with a 72.5% decrease at the lowest effect concentration of 1 µg/L (2.2±0.4), and almost no juvenile production at the highest fenoxycarb treatment (0.3±0.1; Fig. 2). Fenoxycarb also had a significant effect on the proportion of male offspring (Table 1). Under control conditions, around 5% of the parthenogenetic offspring was male. After exposure to 0.1 µg/L (the lowest test concentration) no males were produced in any of the replicates. Exposure to 1 µg/L fenoxycarb or higher concentrations caused a significant increase in male production (Fig. 3; Table 1).

Effects on ephippia production

Production of ephippia showed a (marginally significant) effect of fenoxycarb treatment, with a tendency for a lower number of ephippia per surviving female at the higher fenoxycarb concentrations (Table 1). Under control conditions 0.8±0.1 ephippia were produced per female, which is equivalent to about one ephippium every ten days from the start of production. When exposed to fenoxycarb, ephippia production was between 17.1-47.0% lower (Fig. 2). In a few replicates of the higher fenoxycarb treatments, no ephippia were produced at all. However, even under control conditions, there was quite some variation in ephippia production, and none of the post hoc comparisons among treatments was significant.

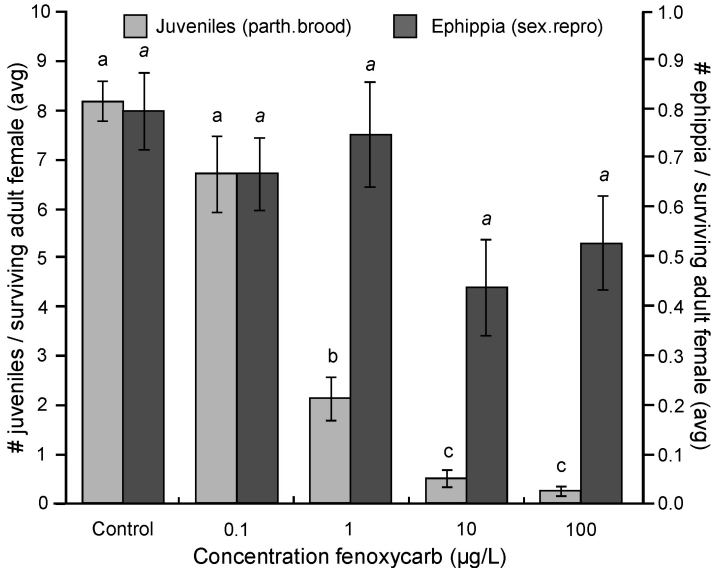

Fig. 2 Effects of fenoxycarb exposure during the sexual phase of the reproduction cycle. Light grey bars represent the total number of juveniles produced per surviving adult female (average, n=9; parthenogenetic offspring). Dark grey bars represent the total number of ephippia produced per surviving female over the course of the experiment (average, n=9; sexual reproduction). Distinct letters on top of the bars indicate significant differences ($p < 0.05$, 1-way ANOVA, Tukey´s HSD post-hoc test; italic letters are used for ephippia production).

Table 1. Results of 1-way ANOVA and generalized linear models, testing for the effects of fenoxycarb exposure on respective endpoints throughout the experiment. The categories in the left column refer to the different phases of the experiment as indicated in Fig.1.

Category	Endpoint	df	F/Chi^2	p-value
1A	Ephippia / surviving female	5	2.45	0.047 *
1B	Juveniles / surviving female	5	69.46	< 0.001 *
1B	Sex ratio offspring	5	135.91	< 0.001 *
1A+B	Survival adults	5	8.28	0.142
2A	Dormant egg volume (µm^3)	5	0.74	0.593
2A	Filling ephippia (%)	5	1.56	0.906
2B	Hatching dormant eggs	5	16.98	0.005 *
2C	Survival hatchlings	5	7.01	0.220
2C	Age maturity hatchlings (days)	5	1.48	0.202
2C	Size 1st brood hatchlings	5	0.46	0.808

Effects on filling, egg volume and hatching of produced dormant eggs

Filling percentages ranged from 80.0-86.5% (Table 2), meaning that most ephippia contained two eggs. There were no significant differences in filling percentage between ephippia collected from control or fenoxycarb treatments nor was there an effect of fenoxycarb treatment on egg volume (Table 1). We did find a significant effect of fenoxycarb exposure on hatching of the isolated dormant eggs (Table 1). From the controls 44.8% of the eggs hatched, whereas 68.8% of eggs hatched from the 1 µg/L fenoxycarb treatment. This was the only treatment that was significantly different from the controls. No dose-related increase or decrease in hatching success was observed.

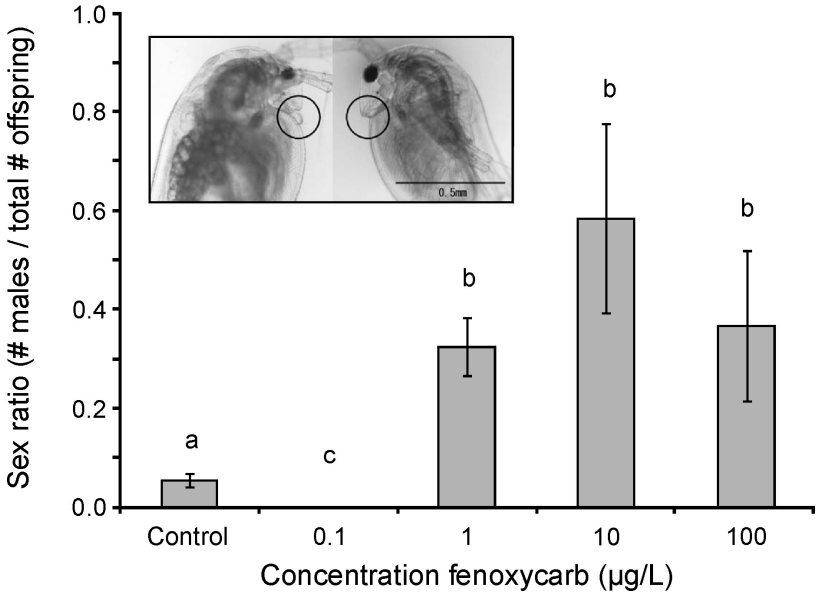

Fig. 3 Effects of fenoxycarb exposure on sex ratio (number of males divided by the total number of juveniles per replicate; average, n=9) of parthenogenetic offspring in *D. magna*. Distinct letters on top of the bars indicate significant differences ($p < 0.05$, generalized linear model, followed by sequential Bonferroni-correction). The photograph in the top left corner shows a 24-hour old male (left) and female (right) of *D. magna* (adapted from Tatarazako et al., 2007), indicating the sexual difference in length and morphology of the first antennae.

Table 2. Summary of the effects of fenoxycarb exposure during the sexual reproductive phase on the produced dormant eggs (endpoints related to Fig. 1, categories 2A-C): quality of ephippia (filling and hatching percentage) and hatchlings (survival and life history traits).

Treatment	Filling (%)	Hatching (%)	Survival (%)	Age maturity mean(±SE)	Size 1st brood mean(±SE)
Category	2A	2B	2C	2C	2C
Control	85.3	44.8	100.0	9.0(±0.3)	14.2(±1.4)
Fenoxcyarb 0.1 µg/L	80.0	42.9	95.0	8.8(±0.3)	11.4(±1.6)
Fenoxcyarb 1 µg/L	86.5	68.8	100.0	8.8(±0.2)	13.1(±1.4)
Fenoxcyarb 10 µg/L	84.8	33.3	100.0	8.9(±0.3)	13.5(±1.6)
Fenoxcyarb 100 µg/L	83.3	48.0	90.0	9.0(±0.6)	13.1(±1.3)

Effects on survival and performance of hatchlings

We did not observe significant effects of fenoxycarb exposure during dormant egg formation on survival of the hatchlings or life history variables of the hatched individuals (age at maturity, size of first brood; Table 1). Hatchling mortality was less than 10% in all treatments. Age at maturity ranged between 8.2 and 9.0 days, and size of first brood ranged from 11.4 to 14.2 eggs per adult female (Table 2).

Discussion

Most ecotoxicological studies using *Daphnia* as a model organism focus on the asexual part of their reproduction cycle. In the present study, we focused not only on acute mortality and sex ratio effects of exposure to the juvenile hormone analogue fenoxycarb during the sexual reproductive phase, but also on the quality of the resulting dormant eggs.

Effects of fenoxycarb on number and sex of offspring

Exposure to fenoxycarb concentrations of 1 µg/L and higher significantly decreased parthenogenetic reproduction and the production of ephippia, while increasing the fraction of male offspring and lowering the production of ephippia. These results are broadly in line with findings from three previous studies, all using different model toxicants. Shurin and Dodson (1997) noted a decrease in ephippia production in *D. galeata mendotae* after exposure to the surfactant nonylphenol, but they did not observe an effect on sex ratio. Similar results were found by Saika et al. (2006), for the exposure of *D. magna* to the marine biocide tributyltinoxide. The concentrations used in these two studies also caused significant mortality, which obscured interpretation of the results. Olmstead and LeBlanc (2001) studied the effects of methoprene (a juvenile hormone analogue like fenoxycarb) exposure on *D. magna* and observed a significant decrease in ephippium production (with around 70% at 10 and 50 µg/L methoprene) and an increase of male offspring production with more than 20%. Despite slightly different test conditions in these studies, test organisms under control conditions produced about one ephippium every ten days (0.083 and 0.090 ephippia/female/day in Olmstead and LeBlanc, 2001 and Saika et al., 2006, respectively), which is similar to the results found in our study (0.088 ephippia/female/day).

In combination, the results of these studies indicate that different types of chemicals that are characterized by different modes of action are able to affect the sexual reproductive phase in *Daphnia*. Olmstead and LeBlanc (2001) postulated that it are environmental stimuli that initiate the sexual reproductive phase, but once induced, juvenile hormone and their analogues are able to interfere with the timing and production of male offspring and ephippia. Indeed, both methoprene and fenoxycarb affected parthenogenetic as well as sexual reproduction. There was, however, a difference in effects of the two chemicals: while methoprene stimulated the production of parthenogenetic offspring at the expense of ephippium production, fenoxycarb caused a decrease in both parthenogenetic and ephippial production.

Effects of fenoxycarb on quality of ephippia

In earlier work, we have shown that dormant eggs can be impacted by exposure to pesticides ($EC_{50\text{-}hatching}$ fenoxycarb = 1,300 µg/L), with negative effects on survival and life history characteristics of the hatchlings (Navis et al., 2013; Chapter 2). In this earlier study exposure took place during the hatching process of the dormant eggs, while in the current study we have exposed adult *D. magna* during the sexual reproductive phase and removed and rinsed the ephippia soon after they were deposited as we wanted to quantify the impact of exposure of the mothers not the ephippia themselves. Although this affected the quantity of ephippia produced, there were no significant effects on the quality of the dormant eggs in terms of filling percentage and the volume of the eggs. There were also no significant differences in survival and life history variables between hatchlings from control versus fenoxycarb treatments. This indicates that even though exposed females are able to produce only a reduced number of ephippia, the viability of the dormant eggs is not negatively impacted by fenoxycarb exposure up to 100 µg/L. In the current experimental setup, we could not expose test organisms to higher fenoxycarb concentrations without causing mortality. In the rotifer *Brachionus plicatilis*, hatching was reported to be the most sensitive endpoint in their life-cycle when dormant eggs were exposed to diazinon (an organophosphate pesticide) during dormant egg production (Marcial et al., 2005; Marcial and Hagiwara, 2007).

Conclusions and ecological relevance

A growing body of research, including the present study, indicates that IGR´s like fenoxycarb can disrupt different parts of the life-cycle in *D. magna*. Previously reported effect levels for parthenogenetic reproduction and offspring sex ratio (male induction) under standard test conditions (according to OECD GD 211; OECD, 2012), were lower than the effect levels found in this study: under long-day photoperiod and non-crowding conditions, male offspring was produced at 0.1 µg/L (Tatarazako and Oda, 2007) and the 21-day $NOEC_{parth.reproduction}$ was 0.002 µg/L (EFSA, 2010). This likely reflects that effects of insect growth regulators may differ among clonal lineages and depend on environmental conditions during exposure (Oda et al., 2007; Lampert et al., 2012). This variation complicates the translation of laboratory test results to impacts at the community level in natural ecosystems.

In addition, in natural systems exposure to toxicants can occur during different points in the complex life-cycle of *Daphnia* (e.g. during exponential growth, during the onset of sexual reproduction, when eggs are dormant in the sediment, or at the start of the growing season when hatching occurs). Disturbances of the sexual reproductive phase in the life cycle of *D. magna,* through changes in offspring sex ratio and a decrease in dormant egg production could lead to a reduction in short-term population growth (active aquatic phase) as well as affecting the size and buffering capacity of the dormant egg bank (dormant phase).

Acknowledgements

This research was funded by a Ph.D. grant of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen). The authors would like to thank Adinda Putman for her assistance with the field sampling, optimization of the experimental design and for providing the *Nannochloropsis limnetica* stock culture, and Ruben Cardoen for practical assistance during the laboratory experiments.

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PART II: COMMUNITY LEVEL

CHAPTER 5

Poisoned through dormancy? Testing the effects of pesticide exposure on active and dormant zooplankton communities in a long-term outdoor mesocosm experiment

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Unpublished manuscript

Abstract

Mesocosm studies are often used in higher-tier risk assessment of pesticides, since these artificial ecosystems allow for testing of specific hypotheses under controlled conditions. Although experimental conditions in mesocosms can be indicated as semi-natural, not all community processes are generally taken into account in exposure experiments. Zooplankton communities rely on a dormant egg bank for long-term survival, but effects of pesticides on dormant egg bank dynamics are currently not included in higher tier mesocosm studies. The aim of this study was to assess the impact of repeated carbaryl exposure in a two year outdoor mesocosm experiment, on both the active and dormant phase of zooplankton communities. In addition, we wanted to assess if effects on the dormant community would lead to changes in the active community during the subsequent growing season. Both a pesticide and a dormant egg bank treatment were included in our experiment to be able to test specifically for effects of pesticide exposure on newly produced dormant eggs (i.e. “new” egg bank) as well as on dormant eggs already present in the sediment fraction (i.e. “old” egg bank). We observed significant negative effects of pesticide exposure on abundances of the established active communities. This was not reflected in the composition of the dormant egg bank, since no effects of carbaryl exposure were observed on taxon richness and abundances of communities hatching from the sediment. We did, however, observe effects of dormant egg bank treatments: taxon richness was significantly lower in the “new” egg bank treatments than in the “old” egg bank treatments, indicating that some taxa were better adapted at surviving and reproducing in these artificial ecosystems than others. No differences were observed in total zooplankton hatchling abundances between the “old” and “new” egg bank treatments, suggesting that after two growing seasons zooplankton populations could produce a sufficient quantity of dormant eggs, to equal hatching from the “old” egg banks. Also, we did not trace significant differences in taxon richness and zooplankton hatchling abundance between the “old” egg bank treatments at the start and end of the experiment, indicating that a mixed, persistent egg bank, can buffer for a reduced input of new dormant eggs. The observed patterns using the current experimental setup, did not show significant impacts of pesticide exposure on the dormant phase. This is however only a first study, specifically designed to assess effects on benthic dormant communities and their coupling to active pelagic communities. Other pesticides, that do show effects on hatching of dormant eggs under laboratory conditions, or different exposure scenarios, might have significant effects on the production, viability, or hatching process of dormant eggs in (semi-)natural systems. Further research into the effects of pesticides on dormant egg bank dynamics is needed to improve our understanding of the long-term effects of pollution on aquatic ecosystems and their potential for recovery.

Introduction

Over the past decades, a continued human population growth accompanied by a sharp increase in food demand, has put a lot of pressure on the agricultural sector to increase crop yields. Since global productive arable areas are limited, fertilizers, pesticides and new crop strains have been increasingly used to boost crop production (Tilman et al., 2002). In the European Union alone, around 280 million kilos of pesticides are used annually (sales of active ingredients 2010; ECPA, 2011). By spray drift, run-off and leaching, a fraction of these pesticides ends up in aquatic water bodies in or surrounding agricultural areas, thereby potentially affecting also non-target organisms (e.g. Lahr et al., 2000; De Schampheleire et al., 2007; Maltby and Hills, 2008).

To ensure safe use, pesticides are evaluated for their impact on the environment as well as on human health, before they can be introduced on the European market (according to EC Regulation No 1107/2009; European Parliamant, 2009). Typical environmental risk assessments for pesticides follow a tiered approach. For the first tier of the assessment, standard aquatic and terrestrial ecotoxicity tests are used, performed according to specific (OECD) test guidelines (Bradbury et al., 2004; EFSA, 2013). Typically, single species tests are performed on representatives of three trophic levels: algae, invertebrates and fish (Walker, 2014). If this first screening indicates potential concerns, registrants are required to demonstrate no unacceptable impact on non-target organisms under realistic field conditions (European Parliamant, 2009). However, field studies are very complex, the causality of effects is often difficult to determine and interpretation of data is challenging (EPiF, 2005; Köhler and Triebskorn, 2013). Therefore, in many cases, higher tier studies use artificial ecosystems (e.g. micro- or mesocosms) as surrogates for actual field testing or surveys (Campbell et al., 1999; De Jong et al., 2008). Mesocosm studies allow testing of specific hypotheses, under semi-natural, controlled conditions. Compared to single species ecotoxicity tests, mesocosm (and field) studies show more ecological realism and allow to consider species interactions, indirect effects and the potential for communities to recover from pesticide exposure (Boxall et al., 2002; Solomon and Sibley, 2002). This is very important, since the long-term effects of pesticide exposure regimes in natural systems, depend on the sensitivity and recovery rate of impacted communities (Barnthouse, 2004). Recovery from exposure is assumed once control and exposed communities are no longer significantly different (Knauer and Hommen, 2012; EFSA, 2013). Van Wijngaarden et al. (2005) reviewed 20 years of data from mesocosm studies testing neurotoxic insecticides and concluded that in general no observed effect concentrations in these artificial ecosystems ($NOEC_{eco}$) were about a factor 10 above predicted no effect concentrations (PNECs), based on first tier screening studies.

While sediment from natural ponds is often used as substrate in aquatic mesocosm experiments, sampling efforts in these studies focus only on the active phase of the aquatic communities (Brock et al., 2000; De Jong et al., 2008). However, many invertebrates living in permanent and temporary standing waters produce dormant stages to survive unsuitable periods (e.g. predation, drought, competition, low oxygen and temperature conditions) (Fryer, 1996; Alekseev and Lampert, 2001; Slusarczyk et al., 2005).

These dormant stages accumulate in the sediment to form dormant egg banks, from which a variable fraction hatches at the start of each growing season (Hairston, 1996). Unhatched eggs remain in the sediment and can act as long term buffer in case of unfavourable conditions (Brendonck and De Meester, 2003). While this benthic-pelagic coupling has important consequences for the ecology and evolution of the inhabiting zooplankton populations and communities (Gyllström and Hansson, 2004), effects on the dormant phase are generally not taken into account in standard ecotoxicological studies (Angeler and Garcia, 2005).

Several studies have indicated that pesticides and other toxicants can significantly impact dormant life stages, either directly or indirectly and this at several phases of the zooplankton life cycle: the production of dormant eggs (Olmstead and LeBlanc, 2001; Saika et al., 2006; Chapter 4), and hatching and survival of dormant eggs (Angeler et al., 2005; Raikow et al., 2006; Navis et al., 2013, 2015; Chapter 2, 3). All of these studies were performed under laboratory conditions, assessing species or population level effects rather than effects at the zooplankton community level. Angeler et al. (2006) did focus on community level effects, testing the effects of fire retardant exposure on invertebrate hatching from wetland sediments, in a laboratory setup. They observed significant negative effects of toxicant exposure on taxonomic richness and zooplankton abundances. Henri et al. (2014) tested the effects of exposure to mining effluents on hatching from zooplankton dormant egg banks. They observed severe impacts of toxicant exposure (heavy metals) on hatchling abundances.

In this study we tested the effects of the pesticide carbaryl in a two year outdoor mesocosm experiment, on both the active and dormant phase of macrozooplankton communities. We used an exposure concentration within the range of concentrations previously reported to affect active zooplankton communities (Hanazato, 1998; Jansen et al., 2011; Sabine Navis, unpubl. data). We specifically aimed to test whether the pesticide would also impact the dormant community (densities and taxon richness), and therefore included two egg bank treatments to test for; 1) indirect effects via the aquatic community (changes in dormant egg production, or in dormant egg quality; i.e. “new” egg bank treatment), and 2) direct effects on the dormant eggs in the sediment (egg mortality, buffering capacity of the dormant egg bank; i.e. “old” egg bank treatment). In addition, we wanted to assess if effects on the dormant community would lead to changes in the active community during the subsequent growing season. Since the pesticide was applied in summer after the main hatching peak we did not intend to study potential direct effects of carbaryl exposure during the hatching process of the dormant eggs. These aspects were presented in former laboratory studies, focusing on acute and chronic effects of pesticide exposure on development and hatching of dormant eggs (Navis et al., 2013, 2015; Chapter 1, 2).

Material and Methods

Experimental design

As starting material for the mesocosm experiment, we used a mixture of sediment collected from five shallow permanent lakes, all situated in the region around Leuven, Belgium: Langerodevijver (LRV), Oud-Heverlee Noord (OHN), Oud-Heverlee Zuid (OHZ), Oud-Heverlee P (OHP) and Zoete Waters 4 (ZW4). These lakes were selected, because the areas surrounding these lakes are not used intensively (no crops for at least 200 m; Google Earth, images 2009-2010), which was determined to be an important parameter influencing the tolerance of *D. magna* populations to toxicant exposure (Coors et al., 2009). In addition, all lakes are known to contain a high density of cladoceran dormant eggs, including *Daphnia magna* ephippia (Louette et al., 2007; Coors et al., 2009; Tom de Bie, pers. com.). A mixture of sediment from these five ponds was assumed to be a good representation of the regional species pool. All lakes were sampled in early spring (February) 2011, when dormant eggs in the sediment were no longer in the refractory (unresponsive) phase as they had already received a natural cold shock, but before the main zooplankton hatching peak takes place (Vandekerkhove et al., 2005). The top 5 cm of the sediment layer was collected at randomly chosen locations in the littoral zone and transported to the experimental site (Heverlee, Belgium). All sediment was stored for three weeks in a cold and dark room to prevent early hatching of dormant eggs in the sediment. During this storage period, 200 L mesocosms were set up and filled with 160 L of tap water. All mesocosms were covered with a net (mesh size 500 µm) to prevent colonization by invertebrate predators and visits by birds and amphibians. Sediment was only added after one week, when the water in the mesocosms was dechlorinated. An equal amount of sediment from all five ponds was mixed using an industrial concrete mixer, and 20 L of the sediment mixture was next added to each mesocosm (equals a layer of 5 cm sediment on the bottom of each tank).

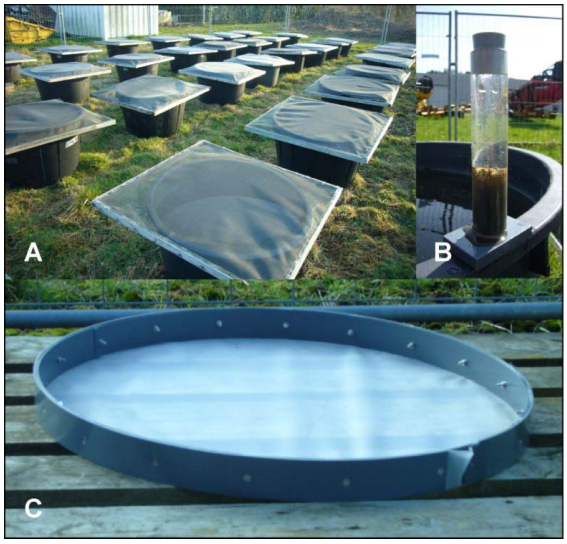

Fig. 1 A) The experimental tanks and setup of the mesocosm experiment, B) sampling device used for taking sediment cores inside the mesocosms, and C) the nets put inside the mesocosms to collect newly produced dormant eggs.

Zooplankton populations were allowed to establish naturally by hatching from the dormant egg bank present in the sediment layer. The first hatchlings were observed in the beginning of April 2011. Once zooplankton communities had established, the 2-year mesocosm experiment was initiated to study effects of carbaryl exposure and two different egg bank treatments in a total of 24 mesocosms (Fig. 1A). In the first year of the experiment (2011) half of the mesocosms received carbaryl (12 carbaryl and 12 control mesocosms; Fig. 2). Carbaryl is a carbamate insecticide inhibiting acetylcholine esterase and causing neurotoxic effects in insects and crustaceans at very low test concentrations (EFSA, 2006). Carbaryl was applied in the summer period (July) with a single pulse of 64 µg/L, nominal concentration using the toxicological approach (direct application and mixing of the water column to ensure equal distribution; Campbell et al., 1999). This test concentration was based on preliminary laboratory experiments with adult and juvenile daphnids in 2 L beakers with sediment (Sabine Navis, unpubl. data), and on previously reported effect levels on zooplankton populations (Jansen et al., 2011) and communities (Hanazato et al., 1998) in outdoor mesocosm experiments. Carbaryl (1-naphthyl methylcarbamate, CAS no. 63-25-2, 99.8% purity, Sigma-Aldrich, Germany) was dissolved in absolute ethanol (purity min. 99.8%, VWR International, France). The stock solution was first diluted into a 1L erlenmeyer, before application to the mesocosms. The concentration of ethanol was the same in carbaryl treated and control mesocosms (0.005% ethanol). This ethanol level was a factor 10 below levels used previously in laboratory experiments, where no statistical differences were observed between the blank and solvent control treatments (Navis et al, 2015; Chapter 1, 3). Measured test concentrations in the water column directly after exposure were 14.1 ± 2.8 µg/L and 38.7 ± 18.8 µg/L (mean ± SE, n = 3) in 2011 and 2012, respectively. After one week concentrations in the water phase had dropped to 0.2 ± 0.1 µg/L and 0.3 ± 0.2 µg/L (mean ± SE, for 2011 and 2012, respectively). Sediment concentrations, one hour after application, were 3.8 ± 1.4 ng/g wet weight and 2.4 ± 0.1 ng/g wet weight (mean ± SE, for 2011 and 2012, respectively).

Three weeks prior to carbaryl application (end of June 2011, before the peak of dormant egg production), a net covering the full diameter of the tanks (diameter 63 cm, 125 µm mesh size; Fig. 1C), was placed into each mesocosm just above the sediment layer to collect newly produced dormant eggs. These nets were used to create two egg bank treatments (i.e. “old” and “new” egg bank, Fig. 2). For the “old” egg bank treatment (12 mesocosms, of which six had previously received carbaryl and six control mesocosms), nets with newly produced ephippia were carefully removed just after the winter period (February 2012). As a consequence, hatching in the subsequent growing season (2012) could only occur from the sediment already present in the tanks since the start of the experiment, as no new eggs were allowed to deposit. Since at each growing season not all eggs in the egg bank hatch as part of a risk spreading strategy (Brendonck and De Meester, 2003), eggs with delayed hatching are still viable and may contribute to future hatching. In this treatment we specifically wanted to assess potential direct effects of carbaryl exposure on the dormant fraction, and the buffering capacity of a mixed persistent egg bank.

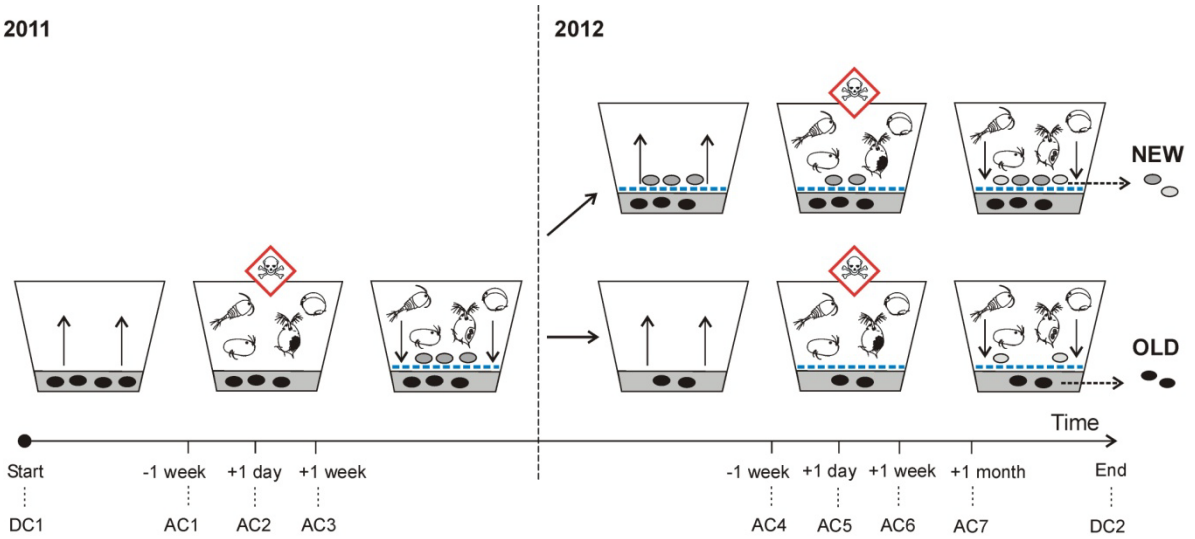

Fig. 2 Experimental design and timeline of the 2-year outdoor mesocosm experiment. Three different phases can be distinguished each year: 1) hatching from the dormant egg bank; 2) the active phase is established and organisms are in the asexual reproductive phase; and 3) at the end of the growing season, a switch is made to sexual reproduction and dormant eggs are produced. In the first year (2011), zooplankton communities were allowed to establish by hatching from the dormant eggs, present in the sediment layer in the tanks (depicted as black dots). In the summer period of the two consecutive years, respective mesocosms received a single carbaryl pulse (indicated with the toxic symbol). Samples of the active phase were taken before and after the pesticide applications, as indicated by AC1 - AC3 for 2011 and AC4 - AC7 for 2012. Dormant egg banks were manipulated by placing a net above the sediment layer in the mesocosms. There were two treatments: "old" in which the active community in 2012 was established by hatching from the sediment layer (black dots), and "new" where only dormant eggs produced during the 2011 growing season (grey dots) could hatch and establish the 2012 active community. Samples of the dormant community were taken at the start and end of the experiment (DC1 and DC2).

After the main hatching peak in spring, nets were placed back in these mesocosms (mid May). For the "new" egg bank treatment (12 tanks; six control and six previously treated with carbaryl), nets were not removed, so hatching in spring could only occur from the newly produced dormant eggs of the first growing season (i.e. "new" egg bank). This treatment was included to assess effects of carbaryl exposure on dormant eggs (quality and quantity) produced during the course of the mesocosm experiment. In July of the second year, a single pulse of 64 µg/L carbaryl was again applied to the respective mesocosms (12 tanks, six replicates coded "carbaryl old" and six replicates "carbaryl new"). No carbaryl was applied to the other mesocosms (12 tanks, six replicates coded "control old" and six replicates "control new"). Timing of application was chosen based on the application period recommended for use of carbaryl containing insecticides in the field (Tessenderlo Kerley Inc., U.S.A).

Sampling

During the two years of the experiment, both the active and dormant communities were sampled repeatedly. In the first year (2011), the active phase was sampled three times (AC1 - AC3; Fig. 2): 1 week before carbaryl application, 1 day and 1 week after application. In the second year (2012), the active phase was sampled four times (AC4 - AC7: Fig. 2): 1 week before carbaryl application and 1 day, 1 week, and 1 month after application. Before sampling the active phase, the water column was gently mixed to homogenize the community. A 5 L beaker was used to collect a total of 20 L water per mesocosm, which was filtered over a 64 µm zooplankton net.

Zooplankton samples were preserved in 70% ethanol and transported to the laboratory, were they were later counted and identified using a stereo microscope (Olympus SZX12). *Simocephalus* and *Alonella* specimens were identified to genus level, all other cladocerans to species level (according to Flössner, 2000). Calanoid and cyclopoid copepods and ostracods were also counted, and included as additional taxa. Subsamples of minimum 300 individuals were counted and densities of the different taxa were expressed as number of individuals per liter mesocosm water.

In addition, at each sampling date, the following environmental variables were measured: pH, conductivity (µS/cm), and oxygen concentration (mg/L). pH and oxygen were measured using a HACH-meter HQ20, conductivity using a WTW-meter Cond 330i set. Algae cover (in %) at the water surface of the mesocosms was estimated, based on visual inspection. For each mesocosm, turbidity and chlorophyll-a levels were also measured (AquaFluor fluorometer, Turner Designs, USA).

The dormant community was sampled twice; at the start and end of the experiment. From each mesocosm, about 200 g of sediment (wet weight) was collected using a small core sampler (height 15 cm, diameter 23 mm; Fig. 1B) at 12 locations in each mesocosm, according to a standardized grid. Sediment was put in 1 L zip lock bags, wrapped in aluminium foil and stored at 4°C in the dark in the laboratory until further use in the hatching experiment. After a storage period of minimum one year at 4°C in the dark, samples from each mesocosm were thoroughly mixed with a spoon and 100 g of sediment per sample (wet weight) was sieved over a 1 mm and 125 µm sieve. The 125 µm – 1 mm sediment fraction (containing dormant eggs) was added to 2 L aquaria (total: 24 mesocosms * 2 sampling moments = 48 aquaria) with diluted ADaM-medium (conductivity 250 µS/cm; Klüttgen et al., 1994). Aquaria were incubated at optimal hatching conditions for Belgian zooplankton populations: 18°C and a photo regime of 16h light : 8h dark (Vandekerkhove et al., 2004b). Hatching was monitored every three days, for a total of 27 days. Newly emerged hatchlings were isolated from the aquaria by carefully filtering the water over a 64 µm sieve. Evaporation of the medium was compensated by adding distilled water. Hatchlings were identified to species level, or when this was not possible to genus level (Flössner, 2000). Zooplankton hatchling abundances and taxon richness were used as a proxy for abundances and composition of the dormant egg bank in the mesocosms.

Statistical analysis

Using univariate statistics, the effects of carbaryl (2011 + 2012) and dormant egg bank (2012) treatment were tested on all measured environmental variables, and various endpoints from the active and dormant communities: taxon richness, total zooplankton abundances and abundances of selected taxa. For all respective endpoints of the active phase we tested if there was a significant effect of carbaryl application and dormant egg bank treatment, using linear mixed effect models, followed by Tukey´s HSD post-hoc tests (for the 2011 data). For the dormant communities, one-way ANOVA´s were used to test for differences at the start of the experiment in 2011 (so before any treatments had been applied) and two-way ANOVA´s were used for egg banks sampled at the end of the experiment in 2012, to test for effects of carbaryl and dormant egg bank treatments (same endpoints as active communities).

Principal response curves (PRCs) were used to analyse community responses to carbaryl and dormant egg bank treatments for both the active and dormant communities (according to Van den Brink and Ter Braak, 1998; Szocs et al., 2015). Like traditional redundancy analysis (RDA), PRCs focus on community differences among treatments, with the advantage that treatment responses over time are adjusted to changes in the control treatment. Abundance data were ln(4x+1) or ln(2x+1) transformed prior to analysis of the active and dormant communities, respectively (for rationale, see Van den Brink et al., 2000). For both the active and dormant community samples "control old" was used as reference treatment and other treatments were plotted relative to this treatment. Species scores were calculated for all taxa. Monte Carlo permutation tests (999 permutations) were used to calculate whether the first PRC axis displayed a statistically significant amount of variation. For the active community phase, separate PRCs were calculated for the two years of the experiment. To test for effects at individual sampling moments, RDAs were computed for each sampling time and a permutation test was run (999 permutations). Subsequently, for sampling moments where a significant treatment effect was observed, Dunnett´s post-hoc tests were used to calculate which treatments were significantly different from the control. All statistical analysis were performed in R statistical software v3.0.2 (The R Foundation for Statistical Computing, 2013), using the *car* package for univariate statistics, the *lme4* and *pbkrtest* packages for the linear mixed effect models, and the *vegan* and *multcomp* packages for multivariate statistics (Oksanen et al., 2013).

Table 1. List of zooplankton taxa encountered in the mesocosms, respectively in the active (aquatic) or dormant (sediment) phase.

Taxon		Abbreviation	Active	Dormant
Bosminidae	*Bosmina longirostris*	Bos_lon	x	x
Chydoridae	*Alona rectangula*	Alo_rec	x	x
	Alonella sp.	Alonella	x	x
	Chydorus sphaericus	Chy_sph	x	x
	Leydigia quadrangularis	Ley_qua	x	x
Daphnidae	*Daphnia magna*	Dap_mag	x	x
	Ceriodaphnia quadrangula	Cer_qua	x	x
	Scapholeberis mucronata	Sca_muc	x	x
	Simocephalus sp.	Simo		x
Macrothricidae	*Iliocryptus sordidus*	-	x[1]	
Sididae	*Sida crystalina*	Sid_cry		x
Copepoda	*Calanoida*	Cal_cop	x	x
	Cyclopoida	Cyc_cop	x	x
Ostracoda	-	Ostr	x	x

[1] one individual detected in a single mesocosm at two sampling dates, only in the active phase (not included in further analysis).

Results

Active phase

We did not observe any significant effects of carbaryl and/or dormant egg bank treatments on the environmental variables measured during the two years of the experiment (Table 2). Samples of the active phase of the mesocosms (AC1 - AC7), contained a total of 12 zooplankton taxa (Table 1). One taxon (*Iliocryptus sordidus*) was excluded from further analysis, since only a single individual was observed at two sampling moments in the same mesocosm.

Taxon richness significantly decreased over the 2011 growing season, both in control and pesticide treated mesocosms (Fig. 3A; Table 2). There was no significant effect of pesticide treatment on taxon richness. Carbaryl had a significant negative impact on total zooplankton and cladoceran abundances in the first year, both one day and one week after application (Fig. 3B+C; Table 2), with *Ceriodaphnia quadrangula* and *Chydorus sphaericus* being the most sensitive species (Fig. 4B+C; Table 2). In the second year we did not observe any significant differences in taxon richness and abundances of zooplankton taxa between carbaryl and control treatments (Fig. 3A+C, Fig. 4A-C; Table 2). We did observe significant effects of carbaryl treatment on total zooplankton abundances (Fig. 3B, Table 2). The dormant egg bank treatment had significant effects on abundances of cladoceran, *D. magna* and *C. sphaericus* (Fig. 3C; Fig. 4A+C; Table 2). Lowest cladoceran abundances were observed in the "carbaryl new" treatment, one day after carbaryl application (Fig. 3C).

Table 2. Results of linear mixed effect models on effects of carbaryl exposure (carb) and dormant egg bank treatment (DEB) during the two years of the experiment on the active communities and measured environmental variables.

Active ZP community	**2011**		**2012**					
(df = 1)	carb		carb		DEB		carb * DEB	
	F	P	F	P	F	P	F	P
Environmental variables								
pH	0.00	0.986	0.31	0.582	1.87	0.186	0.01	0.942
O2 (mg/L)	0.63	0.437	0.05	0.827	3.22	0.088	0.13	0.720
Conductivity (µS/cm)	0.30	0.587	0.33	0.575	1.73	0.204	0.04	0.848
Chlorophyll-a	1.35	0.257	0.60	0.449	0.00	0.957	0.12	0.730
Turbidity	1.71	0.205	0.02	0.890	0.55	0.466	1.23	0.281
Algae cover (%)	2.34	0.140	2.25	0.150	0.10	0.758	0.00	0.970
Active ZP community								
Taxon richness	0.04	0.843	0.43	0.521	0.33	0.573	0.18	0.672
Total ZP abundance	20.24	< 0.001 *	5.83	0.025 *	2.09	0.164	0.98	0.331
Cladoceran abundance	22.99	< 0.001 *	1.45	0.242	7.83	0.011 *	0.00	0.993
Daphnia abundance	0.74	0.399	0.00	0.968	5.74	0.026 *	0.00	0.970
Ceriodaphnia abundance	7.22	0.013 *	3.16	0.091	0.10	0.751	0.00	0.946
Chydorus abundance	21.65	< 0.000 *	0.66	0.426	5.30	0.032 *	0.02	0.877
Copepod abundance	1.71	0.205	0.89	0.357	0.20	0.661	0.54	0.472
Ostracod abundance	1.91	0.181	2.47	0.132	2.79	0.110	2.11	0.162

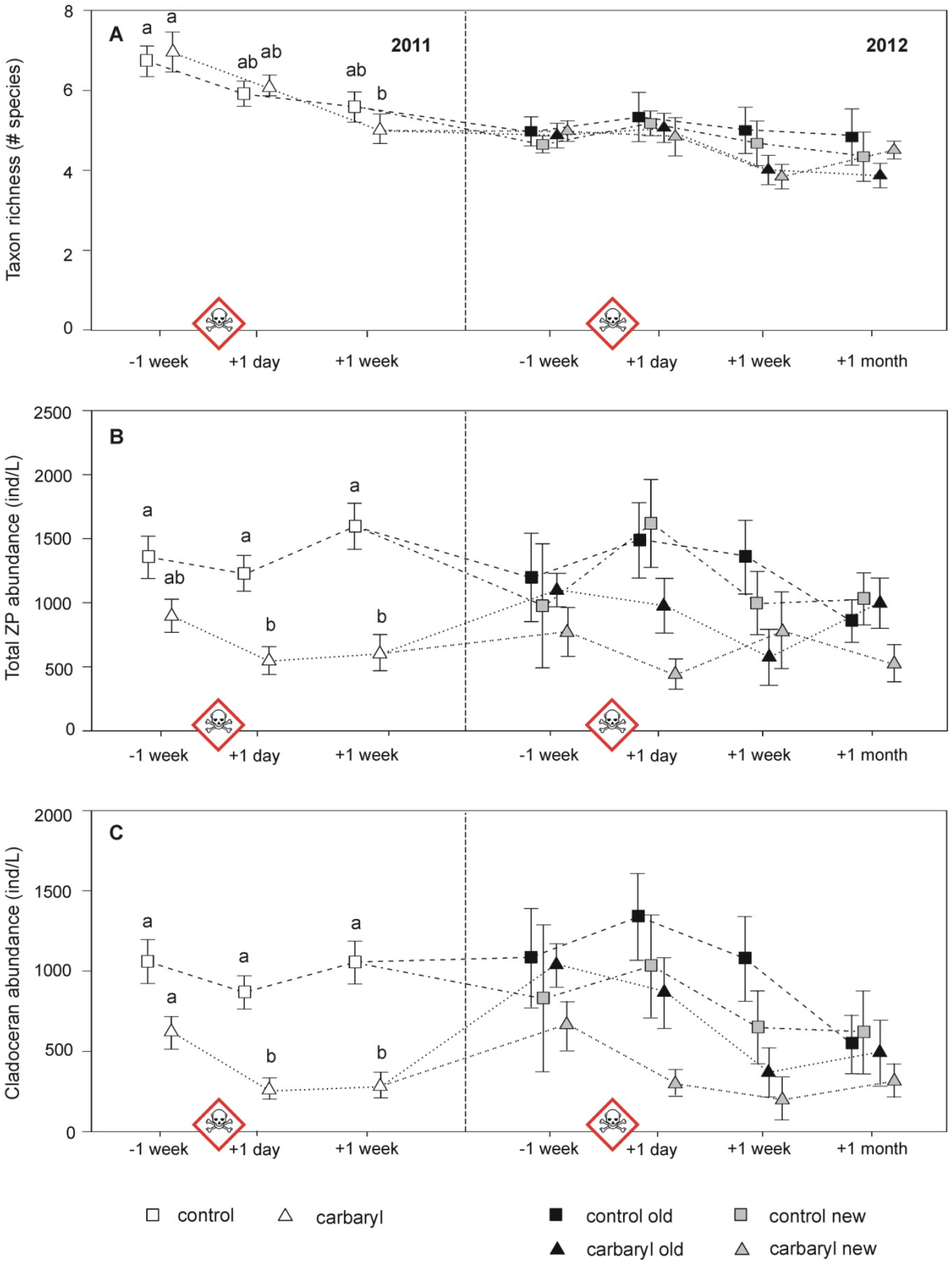

Fig. 3 Effects of carbaryl and dormant egg bank treatments on the active communities: A) taxon richness, B) total zooplankton abundances, and C) cladoceran abundances, at different sampling times during the two years of the mesocosm experiment (average ± 1 SE). Distinct letters in the figures indicate significant differences among treatments and/or sampling times (linear mixed effect models, followed by Tukey´s HSD post-hoc tests , $p < 0.05$; Table 2).

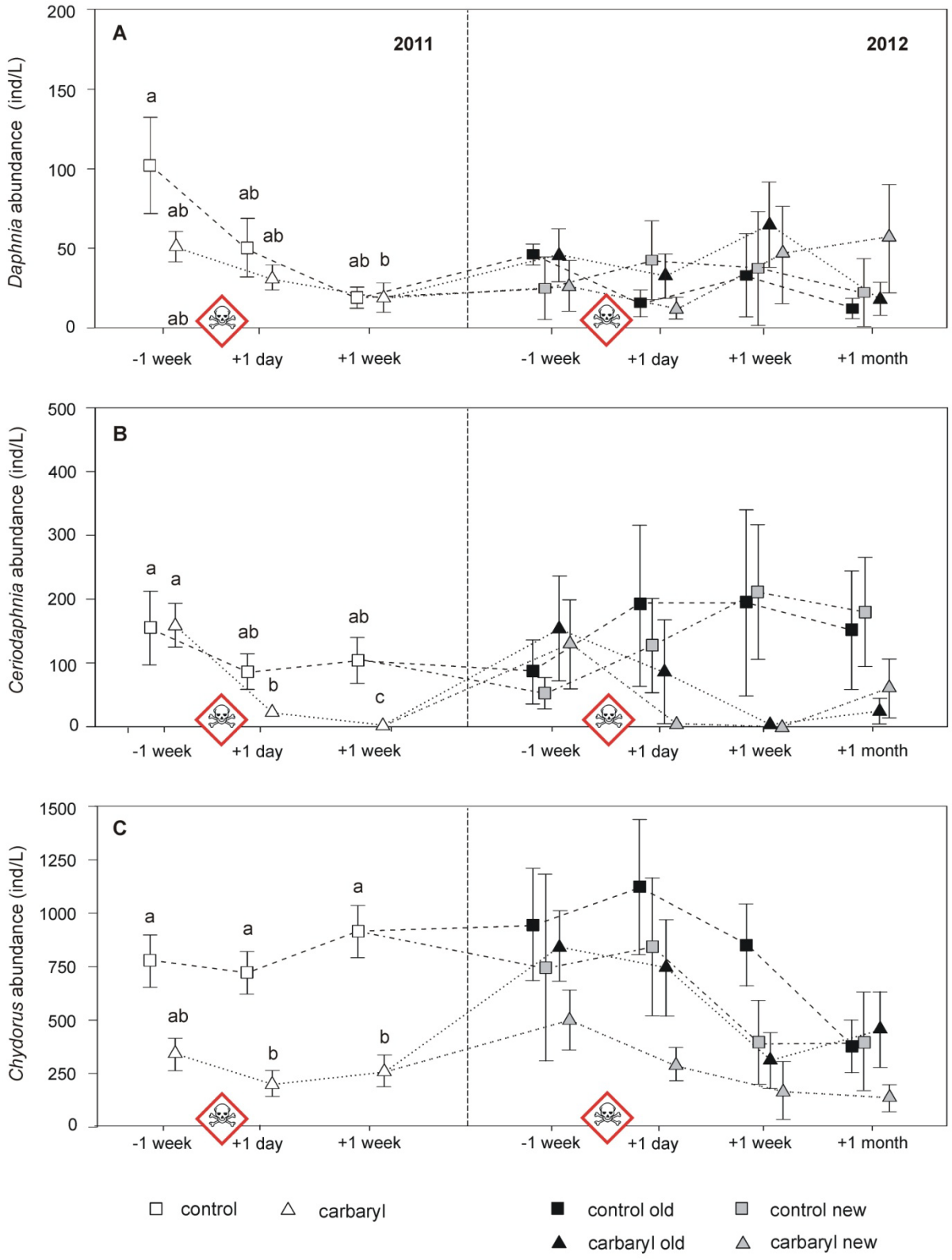

Fig. 4 Effects of carbaryl and dormant egg bank treatments on the active communities: abundances of A) *D. magna*, B) *C. quadrangula*, and C) *C. sphaericus*, at different sampling times during the two years of the mesocosm experiment (average ± 1 SE). Distinct letters in the figures indicate significant differences among treatments and/or sampling times (linear mixed effect models, followed by Tukey´s HSD post-hoc tests , $p < 0.05$; Table 2).

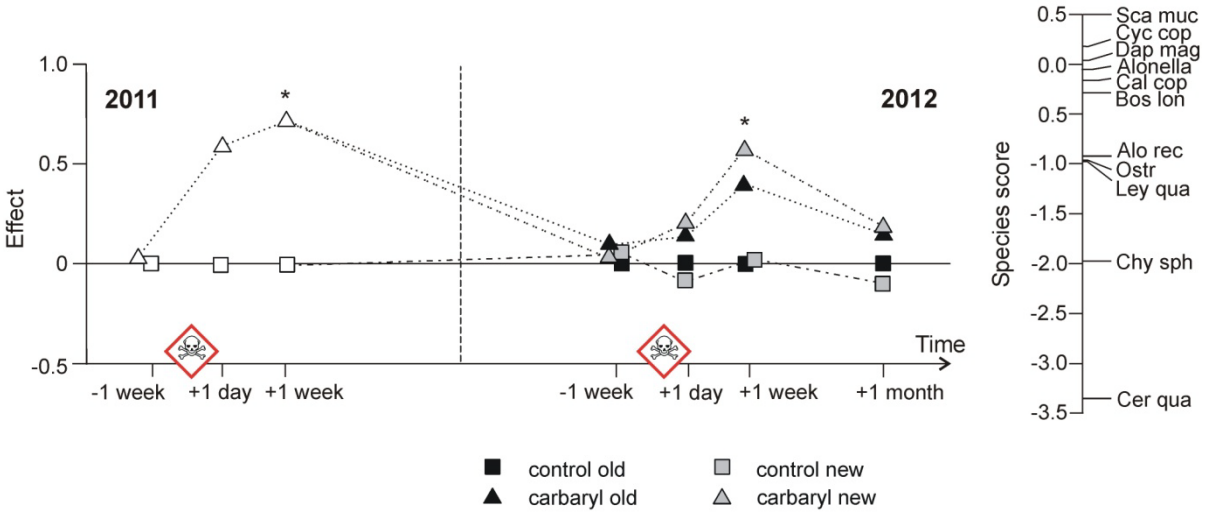

Fig. 5 Principle response curves (PRC1), indicating the active zooplankton community response during the two years of the mesocosm experiment, for both pesticide (control and carbaryl) and dormant egg bank treatments (old and new). All responses were scaled relatively to the untreated "control old" communities. A larger vertical distance from this reference line, indicates a greater divergence. Species scores for all taxa are indicated at the right graph; the higher the score, the more this taxon contributed to the observed pattern (abundances of taxa with a negative score decreased by the treatments, taxa with a positive score increased in abundance). Time and treatment both explain 15% of the variation (PRC analysis; 1st axis explains 47% of the variation; F = 13.98; p = 0.055). * indicate significant differences from the control (old) treatment.

When looking at the zooplankton community composition, carbaryl exposure had a significant effect (F = 10.41, p = 0.001) that was most pronounced one week after application in both years of the experiment (p = 0.003 for AC3 in 2011, and p = 0.033 for AC6 in 2012; Fig. 5). Of all taxa, *C. quadrangula* and *C. sphaericus* were most negatively affected by the carbaryl treatment, whereas *Scapholeberis mucronata* and calanoid copepods showed a positive response to carbaryl treatment (Fig. 5; Table 3, species scores 2011). One week after carbaryl application in the second year, only the "carbaryl new" treatment differed significantly from the control treatment (p = 0.036). One month after carbaryl application, there were no significant differences anymore between the treatments, i.e. the active communities had recovered (p = 0.880; Fig. 5).

Table 3. Species scores for all taxa of the active phase in 2011 and 2012, by Principal Response Curves (PRC1). The higher the score, the more the taxon contributed to the observed pattern (abundances of taxa with a negative score decreased by the treatments, taxa with a positive score increased in abundance).

Taxon	Species score	
	2011	2012
Bosmina longirostris	-0.12	0.00
Alona rectangula	-0.48	-0.02
Alonella sp.	0.02	-0.01
Chydorus sphaericus	-1.89	-1.17
Leydigia quadrangularis	-0.58	-0.67
Daphnia magna	-0.75	1.19
Ceriodaphnia quadrangula	-2.45	-2.94
Scapholeberis mucronata	1.60	-0.28
Calanoid copepods	0.13	-0.55
Cyclopoid copepods	-0.47	0.93
Ostracods	-0.61	-0.91

Dormant phase

A total of 13 zooplankton taxa hatched from the isolated mesocosm sediment (Table 1). Two species, *Simocephalus* sp. and *Sida crystalina*, were retrieved in the hatching experiment but not from the active communities. On the other hand, *I. sordidus* was not encountered in the hatching experiment (but also only in the active community of one mesocosm). There was a significant effect of dormant egg bank treatment on taxon richness, with the lowest number of taxa observed in the "new" dormant egg bank treatments (Fig. 6A; Table 4). Carbaryl application had no significant effect on taxon richness. Dormant egg bank treatment had significant effects on cladoceran and ostracod hatchling abundances (Table 4). Several zooplankton taxa, like *D. magna*, had lowest abundances in the "new" dormant egg bank treatments, whereas abundances of *C. quadrangula* and *C. sphaericus* increased in the "new" egg bank treatments (Fig. 6D-F).

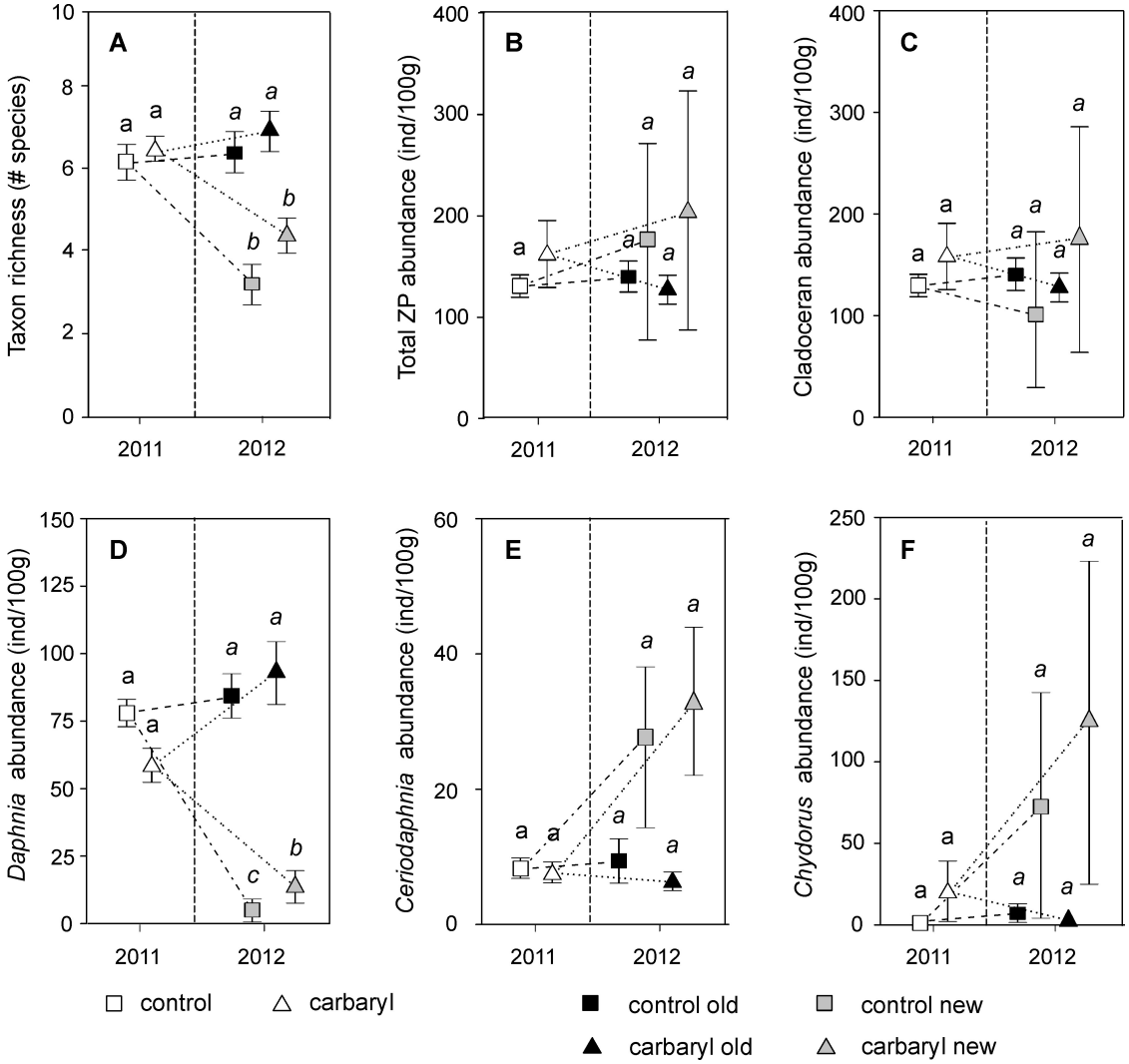

Fig. 6 Effects of pesticide and dormant egg bank treatment on taxon richness and abundances of selected (groups of) taxa, at the start and end of the mesocosm experiment. Abundances represent number of hatchlings per aquarium, containing sediment with dormant eggs from 100 grams of mesocosm sediment, hatched over a 27-day period after incubation (average ± 1 SE). Distinct letters in the figures indicate significant differences among treatments for the two sampling times ($p < 0.05$, Anova´s followed by Tukey´s HSD post-hoc test).

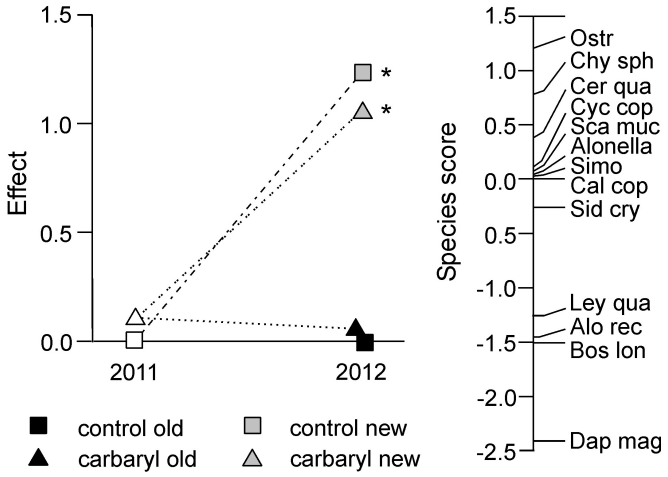

Fig. 7 Principle response curves (PRC1), indicating the dormant zooplankton community response at the start and end of the mesocosm experiment, for both pesticide (control and carbaryl) and dormant egg bank treatments (old and new). All responses were scaled relatively to the untreated "control old" communities. A larger vertical distance from this reference line, indicates a greater divergence. Species scores for all taxa are indicated at the right graph; the higher the score, the more this taxon contributed to the observed pattern (abundances of taxa with a negative score decreased by the treatments, taxa with a positive score increased in abundance). Time explains 13% of the variation and treatment 36% (PRC analysis; 1st axis explains 80% of the variation; F = 22.69, p = 0.001).

Carbaryl application only had significant effects on *D. magna* abundances (Table 4), other endpoints of the hatched community were not affected by the pesticide exposure. Although neither carbaryl, nor dormant egg bank treatment had a significant effect on total zooplankton abundances (Fig. 6B; Table 4), the variation in zooplankton hatchling abundances was much larger in the "new" egg bank treatments, compared to the "old" egg bank treatments. Total zooplankton and cladoceran abundances in "old" egg bank treatments were not significantly different between samples from both years (Fig. 6B+C). When comparing the dormant community composition at start and end of the experiment, a significant effect of dormant egg bank treatment was observed, but not of carbaryl (Fig. 7). At the end, dormant communities of both "control new" and "carbaryl new" differed significantly from "control old" and "carbaryl old" (all p-values < 0.001). Both "new" dormant egg bank treatments ("control new" and carbaryl new") did not differ significantly from each other (p > 0.05).

Table 4. Results of 1-way Anova´s (2011) and 2-way Anova´s (2012) on the effects of carbaryl (carb) and dormant egg bank treatment (DEB) on hatchlings of the dormant phase (sediment collected at DC1 and DC2).

Dormant ZP community	**2011**		**2012**					
(df = 1)	carb		carb		DEB		carb * DEB	
	F	P	F	P	F	P	F	P
Taxon richness	0.41	0.529	3.16	0.090	36.58	< 0.001 *	0.51	0.485
Total ZP abundance	0.25	0.624	0.08	0.776	0.75	0.396	0.26	0.619
Cladoceran abundance	0.20	0.657	0.95	0.341	4.50	0.047 *	1.28	0.272
Daphnia abundance	2.50	0.128	6.35	0.020 *	80.02	< 0.001 *	5.39	0.031 *
Ceriodaphnia abundance	0.33	0.571	0.19	0.669	1.34	0.260	0.97	0.337
Chydorus abundance	0.18	0.669	0.10	0.759	2.85	0.107	0.96	0.340
Ostracod abundance	3.45	0.063	0.00	0.977	26.64	< 0.001 *	0.53	0.475

Discussion

The aim of the present study was to assess the effects of pesticide exposure on pond zooplankton communities in semi-natural ecosystems (mesocosms). To increase the ecological relevance of the experiment, we not only studied effects on the active (pelagic) communities, but also on the (benthic) dormant egg bank, which is an overlooked but crucial component of zooplankton communities. A yearly single pulse exposure of 64 µg/L carbaryl had an expected negative effect on active zooplankton communities. On the other hand, carbaryl had no significant effects on taxon richness and abundance of hatchlings from the dormant egg bank. No effects of carbaryl were observed on quantity of dormant eggs produced during the course of the experiment ("new" egg bank treatment), nor on the dormant eggs already present in the sediment from the start of the experiment ("old" egg bank treatment).

Negative effects of similar levels of carbaryl exposure on active zooplankton communities were also observed in a mesocosm experiment by (Hanazato, 1998). In this experiment, the sensitivity of zooplankton taxa to pesticide exposure was size dependent (larger zooplankton species were more sensitive to insecticide exposure); e.g. *Daphnia* were already affected at 10 µg/L, whereas smaller cladocerans were only affected at 100 µg/L carbaryl. In our experiment, no such sensitivity pattern was observed; on the contrary, species scores even indicate that *D. magna*, the largest cladoceran, was not strongly negatively affected by the carbaryl treatment, in contrast to *C. quadrangula* and *C. sphaericus*, both smaller cladocerans. Smaller sized cladocerans are known to be more sensitive to metal exposure than larger sized taxa (Bossuyt and Janssen, 2005; Vesela and Vijverberg, 2007). This body size-dependent sensitivity was proven to be related to the metabolic rate (especially sodium turnover rate), which is higher in smaller sized organisms, leading to a faster depletion of internal sodium levels (Grosell et al., 2002). Differences regarding relative sensitivity of zooplankton taxa to carbaryl exposure between our study and the studies reviewed by Hanazato (1998), could also be related to differences in timing of carbaryl application during the growing season and/or the presence of predators (*Chaoborus* larvae).

Zooplankton abundances were most impacted one week after carbaryl application, a pattern which was repeated in both years of the experiment. Active communities were recovered one month after the pesticide application. This is in line with Van Wijngaarden et al. (2005), who concluded on the basis of an extensive review of mesocosm studies with insecticides, that recovery of the sensitive species mostly occurred within two months after the last pesticide application. They also concluded that in general no observed effect concentrations in mesocosm experiments ($NOEC_{eco}$) were about a factor 10 above predicted no effect concentrations (PNECs), based on first tier screening studies. Unfortunately, in our study we have only tested a single carbaryl concentration, which does not allow for the calculation of a dose-response relationship or the derivation of effect thresholds, so we cannot make this comparison.

We selected both concentration (64 µg/L) and timing (summer) of carbaryl application to specifically detect the role of the egg bank in community recovery through impact on active communities. The exposure concentration was well below concentrations directly affecting hatching of dormant eggs of *D. magna* under controlled laboratory conditions (Navis et al., 2013; Chapter 2). In addition, the pulse was applied in summer after the main hatching peak took place. Carbaryl has a very short half-life and degrades rapidly in surface waters (hydrolysis half-life of 3.2 hours to 12 days at pH 7 - 9; EFSA, 2006). However, the used carbaryl concentration is known to severely impact survival of adult and juvenile *D. magna,* as well as other zooplankton species, under laboratory conditions (Sabine Navis, unpubl. data; EFSA, 2006; PAN pesticides database). Therefore we expected a reduced survival of the active zooplankton community after carbaryl exposure, which could in turn affect quantity and quality of egg production later in the growing season. Although species abundances were significantly reduced after application with carbaryl in the first year of the experiment, these effects were no longer visible at the start of the next growing season. This suggests that dormant egg banks (both "old" and "new" egg bank treatments) were not affected by carbaryl at concentrations that affected the active communities. This was confirmed by the fact that no effect of carbaryl treatment was observed on hatching of dormant eggs from the sediment collected at the end of the experiment. This underlines the fact that dormant eggs are less sensitive to carbaryl exposure than the active populations, indicating they may buffer for strong impacts on the active communities ("old" egg bank treatment). In addition, surviving individuals had sufficient time for parthenogenetic reproduction after carbaryl exposure in summer, and population densities had recovered by the time of dormant egg production ("new" egg bank treatment), that typically occurs in the fall (Vandekerkhove et al., 2004b). In order to test this hypothesis, genetic analysis of the zooplankton populations throughout the experiment could reveal if indeed carbaryl exposure caused a bottle neck and only a limited set of clones survived the pesticide exposure, and subsequently recolonized the system (Vanoverbeke and De Meester, 2010).

While we did not observe any effects of carbaryl treatment on the dormant phase, dormant egg bank treatment had a significant effect on taxon richness and abundance of hatchlings from the sediment. Taxon richness was significantly lower in mesocosms that re-established from eggs that were produced during the course of the experiment (the "new" treatment), which indicates that not all taxa (a.o. *D. magna*) were successful in producing dormant eggs under experimental conditions. On the other hand, we did not observe any differences in total zooplankton abundances, between hatching from recent ("new") versus the already present persistent ("old") egg banks, indicating that in just two growing seasons zooplankton populations produced sufficient dormant eggs, to equal hatching from the "old" egg banks. This could mainly be related to a few taxa, especially *C. quadrangula* and *C. sphaericus*, that were found to be more successful in the "new" dormant egg bank treatments than in the "old". This pattern could however, also have been established by a higher hatching success of the newly produced dormant eggs, compared to the older eggs present in the sediment.

In addition, there was no significant difference in taxon richness and zooplankton hatchling abundance between the “old” egg bank treatments at the start and end of the experiment, which indicates that a mixed, persistent egg bank, can buffer a strong reduction in dormant egg production (no new eggs were added to the “old” egg bank treatment for two growing seasons). In the current study, we used hatching from the sediment as a proxy for taxon richness and abundances of dormant eggs present in the sediment. We did not evaluate the presence and quantity of any remaining (unhatched) dormant eggs in the sediment fraction. Isolation and morphological determination of dormant eggs remaining in the sediment, could give more information on the size of the dormant eggs banks and the hatching success in the different treatments (Vandekerkhove et al., 2004a).

As a proof of principle, we wanted to evaluate the importance of incorporating egg banks in mesocosm studies, assessing the long-term effects of pesticide exposure on zooplankton population and community dynamics. The observed patterns using the current experimental setup, did not show significant impacts of pesticide exposure on the dormant phase. This is however only a first study, specifically designed to assess effects on benthic dormant communities and their coupling to active pelagic communities. Other pesticides, like fenoxycarb, that do show effects on hatching of dormant eggs under laboratory conditions (Navis et al., 2013, 2015; Chapter 1, 2, 3), or different exposure scenarios, might have significant effects also on the production, viability, or hatching process of dormant eggs in (semi-)natural systems. Further research into the effects of pesticides on dormant egg bank dynamics is needed to improve our understanding of the long-term effects of pollution on aquatic ecosystems and their potential for recovery.

Acknowledgements

This research was funded by a Ph.D. grant of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen) and the KU Leuven Research Fund (Excellence Center Financing PF/2010/07). The authors would like to thank all colleagues (“de Brennies”) for their invaluable assistance at the experimental site during the sampling moments, especially Melissa Schepens. In addition, we would like to acknowledge the lab technicians Geert Neyens and Rony Van Aerschot, who helped with the sediment sampling and practical preparations for the mesocosms. For their contributions to the laboratory hatching experiments, we would like to thank Camille De Raedemaeker and Maarten Goedseels. We are very grateful to Sarah Tilkin for her voluntary help with the identification of the preserved active community samples. And to Joost Vanoverbeke for his help with the statistical analysis.

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GENERAL DISCUSSION

Introduction

Ecotoxicity testing and biomonitoring of aquatic ecosystems generally focus on the active component of invertebrate communities (Jeppesen et al., 2003; Vandekerkhove et al., 2005b; Chiaia-Hernandez et al., 2013). The effects of anthropogenic stressors, such as pollution, on dormant egg bank dynamics have been studied far less (Moest et al., 2015), even though dormant egg banks are crucial for the long-term survival of many aquatic populations and communities (Brendonck and De Meester, 2003; Gyllström and Hansson, 2004). To improve our understanding of the effects of pesticide exposure on dormant egg bank dynamics, we studied different endpoints related to the dormant phase in zooplankton populations and communities, both under controlled conditions in the laboratory, as well as in a semi natural mesocosm environment.

In this section, we first discuss potential scenarios through which pollutants can affect populations of zooplankton. Secondly, we discuss the impact and ecological relevance of these potential scenarios for dormant egg bank dynamics in natural ecosystems. Using the model pesticide fenoxycarb as a case study, we then identify which endpoints in the complex life-cycle of the model organism *Daphnia magna* appear most sensitive to pesticide exposure. Next, we discuss the impact at the zooplankton community level, comparing effects on the active and dormant phase in semi natural ecosystems. And finally, we indicate possibilities and future perspectives of dormant egg bank dynamics research from an ecotoxicological perspective.

Impact of pollution on dormant egg bank dynamics – potential scenarios

As introduced by Angeler and Garcia (2005), anthropogenic stressors can have potential impacts on dormant egg bank dynamics, through one or several of the following scenarios (Fig. 1; General introduction): 1) impact on development and hatching of dormant eggs; 2) effects on hatchling survival and performance in the aquatic phase; 3) impact on dormant stages before activation, causing egg mortality or irreversible disruption of the dormancy-break system; 4) impact on the sexual reproductive phase, leading to (quantitative or qualitative) changes in dormant egg production. In the following paragraphs each of these scenarios is discussed in more detail.

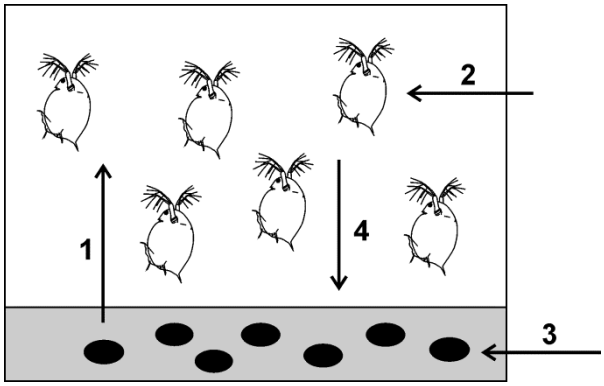


Fig. 1 Potential scenarios in which toxicants can impact dormant egg bank dynamics: 1) impact on hatching process; 2) effects on hatchling survival and performance; 3) impact on dormant stages before activation; 4) effects during sexual reproductive phase. Also combinations of one or more of the above mentioned scenarios are possible.

Impacts on development and hatching

In this thesis we focused specifically on effects of pesticide exposure on development and emergence of *D. magna* dormant eggs using a series of laboratory experiments (**chapter 1-3**; scenario 1). In **chapter 1** we screened five pesticides with a different mode of action, for their impact on development and hatching of *D. magna* dormant eggs. Exposure of dormant eggs to the pesticides simultaneously with incubation under conditions that induced hatching. This scenario is comparable to pesticide applications after ending of the refractory phase of dormant eggs in spring, resulting in high concentrations of pesticides in small water bodies (via spray drift, runoff or drainage) coinciding with a peak in hatching from the dormant egg bank. Our results indicate that all pesticides were able to affect the hatching process, but the type of deformations and effect levels differed between the model pesticides. In **chapter 2** we studied two of the previously screened pesticides in more detail. Carbaryl had no negative effects on hatching up to concentrations almost 1000 times the median effect concentration (EC_{50}) of neonate survival in acute tests. Fenoxycarb, however, had a significant dose-related effect by delaying or completely stopping the hatching process and caused severe deformations in developing individuals at concentrations about twice the acute EC_{50} for neonate mortality.

Similar results were obtained by the few other studies that have assessed effects of pesticides on hatching of *Daphnia* dormant eggs. The biocides sodium hypochlorite and menadione (SeaKleen) affected hatching success of *D. mendotae* dormant eggs at effect levels above those affecting other *D. mendotae* life stages (Raikow et al., 2006, 2007). Other studies focused on different classes of toxicants. Fire retardant treatments significantly negatively affected hatching success of *D. curvirostris* dormant eggs (Angeler et al., 2005). Acid mine drainage (mainly containing heavy metals) severely impacted hatching of, among others, *Daphnia* dormant eggs from sediments (Henri et al., 2014). Moest et al. (2015) is the first study to report an increase in hatching success of *D. longispina* dormant eggs exposed to a mixture of organic contaminants. However, they exposed a large set of ephippia simultaneously, without checking for the presence of unhatched eggs after the experiment. In addition, they only checked for hatching every three days, introducing a large uncertainty in their results. Although not much is known about the physiological basis behind the effects of pollutants on the hatching process (Jiang et al., 2007; Moest et al., 2015), results clearly indicate that various classes of toxicants are able to disrupt the hatching process of *Daphnia* dormant eggs.

The effects of toxicant exposure on hatching of dormant eggs were also assessed for several other zooplankton taxa. Marcial et al. (2005) found that the pesticides diazinon, fenitrothion, methoprene and isoprothiolane affected hatching of rotifer dormant eggs (*Brachionus plicatilis*) at concentrations 2-40 times lower than concentrations affecting population growth and reproductive endpoints. Impacts of heavy metals (copper, lead and cadmium) on hatching of copepod dormant eggs (*Acartia pacifica)* occurred at lower levels than observed to affect survival of benthic adults (Jiang et al., 2007).

For *Artemia*, the results of different studies appear to be inconclusive. Varó et al. (2006) and Sarabia et al. (2003, 2008) reported no adverse effects of metal (mercury and zinc) and pesticide (chlorpyrifos) exposure on hatching of *Artemia* cysts. Bagshaw et al. (1986) and Rafiee et al. (1986), on the contrary, revealed that hatching of dormant eggs was more sensitive to heavy metal (cadmium and zinc) exposure than survival of hatched individuals. This indicates that effects of chemicals on dormant stages during the hatching process differ among and within species as well as among toxicants, depending on their mode of action.

Besides the type of toxicant, also the moment of exposure could determine the (extent of the) effects on the hatching process. In **chapter 3**, we investigated the effects of pesticide exposure on dormant eggs at different embryonic developmental stages and evaluated the potential for bioaccumulation of fenoxycarb in the eggs. Both the impact on hatching characteristics as well as the internal egg concentrations depended on the timing of exposure. Final stages of embryonic development were most sensitive to pesticide exposure and had the highest tissue concentrations of fenoxycarb. The effects of pesticide exposure seemed to be determined by the number of membranes surrounding the embryos during the exposure period. Before light activation, dormant eggs are surrounded by three membranes (Zaffagnini, 1987; Seidman and Larsen, 1979), while after activation these membranes are shed during embryonic development. At the last developmental stage, embryos are only protected by one external membrane and become much more active, allowing a higher influx of the surrounding medium (Davison, 1969) and thus also potential toxicants, into the eggs. Similar results were previously found for parthenogenetic eggs of *D. magna*, where the last embryonic instars of developing eggs were most sensitive to metal exposure (Bodar et al., 1989). However, unlike parthenogenetic eggs, dormant eggs have thick multi-layered membranes (Seidman and Larsen, 1979) that protect them from mechanical damage and digestive enzymes of organisms like fish and birds (Figuerola and Green, 2002). In addition, they are encapsulated in a protective envelope (ephippium; Zaffagnini, 1987). We specifically tested for the degree of protection against pollution provided by the ephippial case (**chapter 3**) and did not observe differences in tissue concentrations of fenoxycarb between decapsulated and encapsulated eggs. This suggests that the ephippial case offered limited or no direct protection against pesticide exposure. Even though dormant eggs show a high tolerance to extreme physical conditions (Mellors, 1975; Radzikowski, 2013), they can hence still be directly affected by chemical pollution.

Effects on hatchling survival and performance

Hatchling survival and performance in the aquatic phase can be impaired by toxicants by means of different pathways (Angeler and Garcia, 2005; Moest et al., 2015; scenario 1, 2). First of all, due to pollutant exposure, environmental conditions in the aquatic phase can become unsuitable for hatched individuals even when pollutants did not directly affect the hatching process itself. Secondly, exposure to toxicants during the hatching process can have chronic effects on survival and performance of hatched individuals. In **chapter 2** we tested this second pathway by following up hatchlings from previously exposed dormant eggs in a life-table experiment until the release of their second brood.

We observed that both model pesticides (carbaryl and fenoxycarb) had significant negative effects on survival and reproduction of the hatchlings, even though carbaryl had no direct negative effects on hatching success. These results indicate that, in addition to inducing mortality of active individuals, pesticides can affect zooplankton communities by altering hatching dynamics and life history traits of hatched individuals. Moest et al. (2015) also observed reduced survival of *D. longispina* hatchlings, when dormant eggs were previously exposed to a mixture of organic contaminants. However, hatchling survival was only assessed every three days, so a reduction in survival could also have been due to toxic effects of the chemical mixture upon individuals after hatching.

Direct impact - mortality of dormant stages

In addition to causing effects during the hatching process, toxicants can also affect the eggs while they are in a state of dormancy (diapause or quiescence), by causing egg mortality or by disrupting or blocking cues for hatching, thereby causing a decrease in hatching once triggered (Angeler and Garcia, 2005; scenario 3). Henri et al. (2014) observed that when sediments exposed to mining effluents were subsequently exposed to control (optimal) hatching conditions, some recovery was observed, but hatching was still significantly impaired. This indicates that not only cues for hatching were blocked, but the effluent also caused direct mortality of (part of) the dormant eggs. Similar results were observed by Angeler et al. (2005), who found a reduction in hatching from wetland sediments after previous exposure to a fire retardant, also indicative of direct effects on the dormant eggs. In **chapter 3** we observed negative effects of fenoxycarb exposure on dormant eggs of *D. magna* shortly before light activation. Preliminary results of another study (S. Navis, unpublished data), indicated that repeated exposure to carbaryl or fenoxycarb of dormant eggs present in the sediment fraction caused chronic effects on survival and performance of hatched individuals. Dormant eggs used in these two experiments had previously been stored in cold conditions, which completed the refractory phase and ensured that diapause was terminated, so the eggs became quiescent and hatching could be induced under favorable conditions (Stross, 1971; Vandekerkhove et al., 2005a). It remains to be tested whether the effects of pollutants on dormant eggs are depending on the type of dormancy (e.g. quiescence versus diapause). Henri et al. (2014) proposed that diapausing eggs could be more tolerant to stressors than quiescent eggs, since quiescent eggs are activated and hence sensitive to external conditions.

Effects during the sexual reproductive phase - dormant egg production

Previous studies that tested the impact of chemical exposure during the sexual reproductive phase on dormant egg production in *Daphnia* (Shurin and Dodson, 1997; Olmstead and LeBlanc, 2001; Saika et al., 2006; scenario 4) observed a decrease in ephippia production with increasing toxicant (nonylphenol, methoprene and tributyltinoxide, respectively) concentration. However, none of these studies tested for effects of toxicant exposure on the quality of the produced dormant eggs, as can be revealed by studying the hatching success, survival and life history characteristics of hatched individuals.

In **chapter 4**, we showed that exposure to fenoxycarb at 1 µg/L or higher concentrations caused a decrease in both parthenogenetic and sexual (dormant) egg production, while inducing the production of male offspring. There were, however, no significant effects of fenoxycarb exposure on the survival and life history characteristics of the hatchlings. This indicates that even though the quantity of dormant eggs was reduced, their quality did not seem to be affected significantly by fenoxycarb exposure. This is contrary to the results of Marcial and Hagiwara (2007), who discovered that for the rotifer *B. plicatilis* hatching rates were severely affected by diazinon when female rotifers were exposed during dormant egg production.

Ecological implications - impact of pollution on dormant egg bank dynamics

Our results of **chapter 1-4** show that pollution can affect dormant egg bank dynamics through all four scenarios discussed. This can have far-reaching consequences for ecological and evolutionary dynamics of zooplankton populations and communities in lakes and ponds. Exposure to toxicants can occur during different phases in the life-cycle of zooplankton species: e.g. during exponential growth, during the onset of sexual reproduction, when eggs are dormant in the sediment, or at the start of the growing season when hatching occurs. Even a short exposure to pesticides during the early stages of embryonic development of dormant eggs can lead to reduced hatching success (fenoxycarb; **chapter 1+2**), a higher proportion of deformed embryos (all five model pesticides; **chapter 1**) and higher mortality and decreased performance of hatchlings (carbaryl and fenoxycarb; **chapter 2**). Reduced hatching rates and performance will reduce population growth rates, which in turn may impact algal growth and fish predation. In addition, disturbances of the sexual reproductive phase, through changes in offspring sex ratio and a decrease in dormant egg production (**chapter 4**) could also lead to a reduction in short-term population growth as well as affecting the size and buffering capacity of the dormant egg bank. These effects might be amplified when the decrease in ephippia production is combined with changes in timing and quantity of males produced. If this leads to a mismatch between male and sexual egg production, this may result in reduced fertilization of dormant eggs. In Box 1 an example is presented to illustrate the potential implications of pesticide exposure at a *D. magna* population level, when pesticide exposure takes place at the start of the growing season, shortly preceding the main hatching peak.

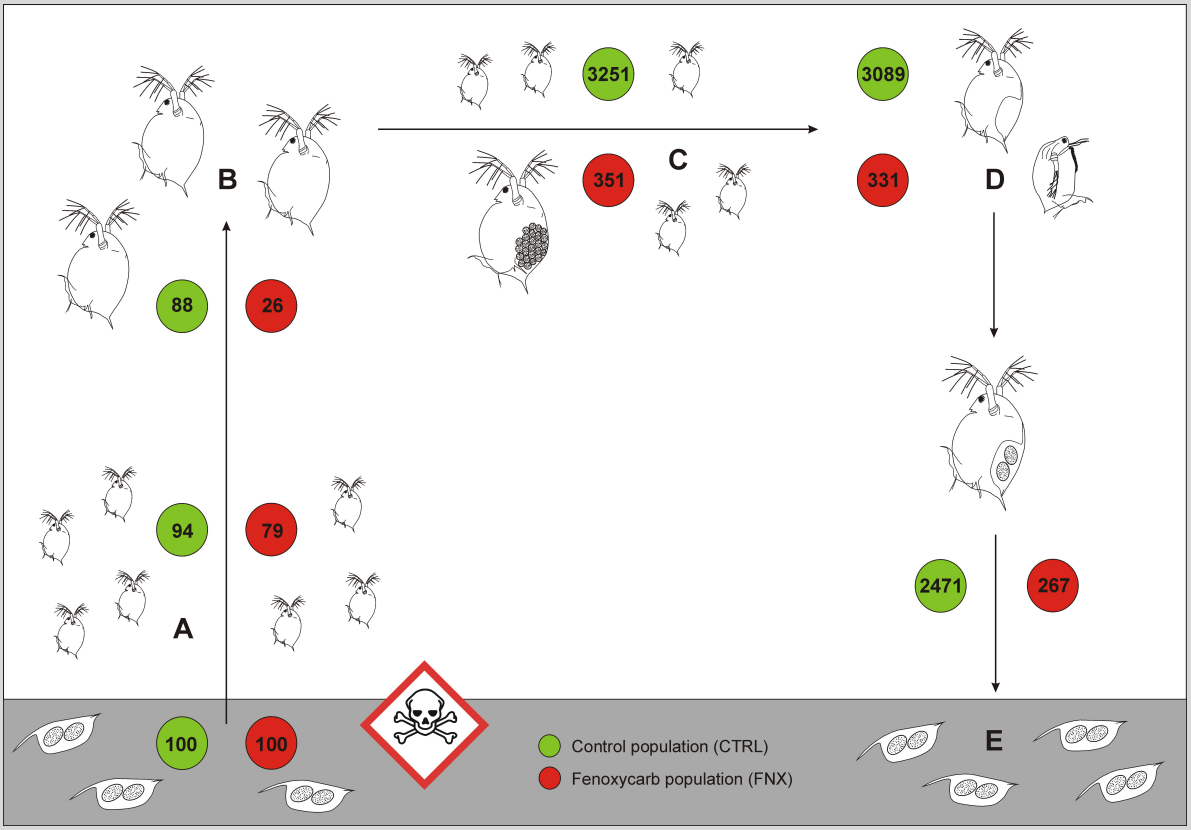
The potential impact of pesticides on dormant egg bank dynamics does not only depend on the application time, but also on the exposure route of the respective pesticides. Pesticides like fenoxycarb are known to dissipate from the water phase quite rapidly and bind to the organic phase of the sediment (Sullivan, 2000; Chiaia-Hernandez et al., 2014). Since dormant eggs can remain viable for decades to centuries (Frisch et al., 2014), exposure to contaminants present in the sediment component can take place over extended periods of time, leading to bioconcentration of pollutants in dormant stages (Wyn et al., 2007; Chiaia-Hernandez et al., 2013; **chapter 3**). Exposure to a mixture of organic contaminants similar to those found in lake sediments, with the potential to bioaccumulate in dormant eggs, was found to affect hatching success of *D. longispina* dormant eggs under laboratory conditions (Moest et al., 2015). Since dormant egg banks integrate genetic variation that has accumulated over many growing seasons (Ellner and Hairston, 1994; De Meester et al., 2006), exposure to pollutants leading to a decrease in size of the dormant egg bank or a reduced contribution of the dormant to the active phase may reduce genetic variation, hence the evolutionary potential of zooplankton populations (Levin, 1990; Brendonck and De Meester, 2003). This could make exposed populations more vulnerable to changes in environmental conditions or anthropogenic stressors.

BOX 1. Example illustrating the potential effects of pesticide exposure at the *D. magna* population level, through effects on the dormant phase.

Scenario: Pulse exposure of 1000 µg/L fenoxycarb shortly preceding the hatching peak in a natural aquatic system containing a dormant egg bank with *D. magna* ephippia.

Effects, besides mortality of the already established neonates (72.5%; unpublished data):

A) Reduction in hatching of dormant eggs with 15.2% (from 93.8% to 78.6%; Fig. 2, Chapter 2). Assuming a starting situation with 100 dormant eggs, 94 individuals will hatch in the control population (CTRL) and 79 in the population exposed to fenoxycarb (FNX).
B) Due to fenoxycarb exposure, in the FNX population 79.4% of the hatchlings will have severe deformations, and survival to maturity will be limited to 33.3%, versus 93.3% in the CTRL population (Fig. 3, Chapter 2). This equals 88 surviving adults in CTRL and 26 in FNX.
C) In addition, performance (r) of surviving individuals in the FNX population will be reduced, since r (parthenogenetic population growth) will be 0.225 instead of 0.360 (Fig. 4, Chapter 2). After three generations, with each three reproductive events, this will lead to a CTRL population size of 3251 individuals and an FNX population of 351 organisms (reduction in population size of 89.2%; calculations according to Birch, 1948).
D) Assuming 95% of the established populations is female (cf. sex ratio under control conditions; Fig. 3, Chapter 4), there will be 3089 females in the CTRL and 331 in the FNX population.
E) If these females produce 0.8 ephippia over a 21 day period (Fig. 2, Chapter 4), there will be 2471 ephippia produced in the CTRL vs. 267 ephippia in the FNX population.



Most sensitive endpoints in the life-cycle of *D. magna* – a case study with fenoxycarb

Although we know that different classes of pollutants are able to affect dormant egg bank dynamics, it remains unclear which endpoints are most sensitive and whether endpoints related to the dormant component are more sensitive than endpoints traditionally tested in standardized ecotoxicity tests. So far we have tested five different model pesticides of which one, fenoxycarb, caused clear dose-related negative effects on development and hatching of dormant eggs (**chapter 1-3**). To compare the sensitivity of the different endpoints for this pesticide, we will present a comparison of effect levels for all endpoints of the life-cycle in *D. magna* that are available, either from this thesis, or from literature (Table 1).

Lowest effect levels of fenoxycarb in *D. magna* are observed for parthenogenetic reproduction: a reduction in the number of produced juveniles was already observed in the ng/L range (21d NOEC = 1.6 ng/L; EFSA, 2010). Concentrations starting from 0.1 µg/L caused a switch in the sex ratio of parthenogenetic offspring (Tatarazako and Oda, 2007) as significantly more males were produced. Effect levels for sex ratio do seem to differ among clonal lineages: the OECD (2008) reported a factor of 20 difference between different clones tested at various laboratories under the same standardized test conditions. Although we tested for effects on sex ratio under conditions inducing sexual reproduction (crowding, short-day photoperiod), the observed effects levels for sex ratio (LÒEC = 1 µg/L; **chapter 4**) are similar to those reported under standard test conditions (test organisms individually in beakers, long-day photoperiod). Effects levels on embryonic development of both parthenogenetic and dormant eggs are also in this same range (LOEC = 0.1 µg/L; **chapter 1** and EC_{50} = 2.7 µg/L; Mu and Leblanc, 2004). At these low exposure levels, fenoxycarb induced deformations in the tail spine (curved). Nevertheless, this type of deformations is reversible when hatchlings are reared under optimal conditions (Mu and Le Blanc, 2004).

More severe deformations of the carapax en antennae were observed in dormant egg embryos and hatchlings at concentrations of 1000 µg/L and higher (**chapter 1 + 2**). This type of deformations was irreversible and lead to a reduction in survival and performance of the hatched individuals (LOEC = 500 µg/L, also the lowest concentration tested for chronic effects; **chapter 2**). Hatching of dormant eggs, either when exposure took place before or after light activation, was among the least sensitive endpoints (EC_{50} hatching = 1300 µg/L; **chapter 2**), about a factor 2.5 higher than concentrations causing acute mortality in neonates (EC_{50} = 500 µg/L; EFSA, 2010).

To conclude, for fenoxycarb the endpoint from a standard ecotoxicity test (in this case effects on parthenogenetic reproduction) that is currently being used in the risk assessment process, appears to be the most sensitive endpoint. However, pesticides like fenoxycarb are able to affect development and hatching of dormant eggs, as well as have more chronic effects on survival and life-history characteristics of hatched individuals. For fenoxycarb these effects occurred at or just above effect levels for neonate toxicity.

Table 1 Overview of effects of fenoxycarb exposure on endpoints in the life cycle of *D. magna*. Numbered scenario´s refer to scenario´s modified from Angeler and Garcia (2005), as described in the previous paragraphs.

Scenario	Endpoint	Test duration	Effect level	Toxicity (µg/L)	Reference
	Neonate mortality/immobility	48 h	EC_{50}	500	EFSA (2010)
	Neonate mortality/immobility	48h	EC50	210 - 860	OECD (2008)
	Parthenogenetic reproduction	21 d	NOEC	0.002 - 3.2	EFSA (2010)
	Parthenogenetic reproduction [1]	21d	LOEC	1	Chapter 4
4	Sex ratio (male induction)	21d	EC_{50}	0.45 - 10	OECD (2008)
4	Sex ratio (male induction)	21d	LOEC	0.1	Tatarazako and Oda (2007)
4	Sex ratio (male induction) [1]	21d	LOEC	1	Chapter 4
1	Embryo toxicity parthenogenetic eggs (malformations: curved tail spine)	3d	EC_{50}	2.7	Mu and LeBlanc (2004)
1	Embryo toxicity parthenogenetic eggs (malformations: curved tail spine)	10d	LOEC	0.1 [2]	Chapter 1
1	Embryo toxicity dormant eggs (malformations: curved tail spine)	10d	LOEC	0.1 [2]	Chapter 1
1	Embryo toxicity dormant eggs (malformations: carapax + antennae)	10d	LOEC	1000	Chapter 1 + 2
4	Dormant egg production	21d	LOEC	n.d. [3]	Chapter 4
1	Hatching dormant eggs (exposed during hatching process)	10d	EC_{50}	1300	Chapter 1 + 2
3	Hatching dormant eggs (exposed before hatching process)	10d	LOEC	1000	Chapter 3
2	Hatchling survival	21d	LOEC	500 [2]	Chapter 2
2	Hatchling performance	21d	LOEC	500 [2]	Chapter 2

[1] under conditions inducing sexual reproduction

[2] lowest tested concentration

[3] significant effect of fnx, but post-hoc comparisons not significant

In addition to the traditional acute and chronic *Daphnia* ecotoxicity tests, standardized hatching experiments (**chapter 1 – 2**) could be used as a fast and efficient method in screening toxicants for effects on development and hatching of dormant eggs, similar to in vitro assays currently being used to screen for effects on parthenogenetic eggs (Palma et al., 2009; Sobral, 2001; Abe et al., 2015). Since we have only screened five pesticides for effects on development and hatching, and only two pesticides for more chronic effects, testing of other classes of toxicants is highly recommended. Since there are indications that even pesticides that do not directly affect the hatching process are able to impact survival and life history characteristics of hatchlings (**chapter 2**), more research is needed to determine the long-term ecological impact of exposure to environmental relevant concentrations of pesticides, under different exposure scenarios.

Community level effects

In the previous paragraphs, we have focused on effects of pollutants on dormant life stages at the population level (*D. magna*). However, in natural systems the situation is (even) more complicated, since other biotic and abiotic factors play a role in determining the direct and indirect effects of pollution, such as climatic conditions, species traits and interactions (e.g. Solomon and Sibley, 2002; Relyea and Hoverman, 2006; Liess et al., 2008). While field studies are ecologically most relevant, they are also very complex, the causality of effects is often difficult to determine and interpretation of data is challenging (EPiF, 2005, Köhler and Triebskorn, 2013). Therefore, in many cases higher tier studies use artificial ecosystems (e.g. micro- or mesocosms) as surrogates for actual field testing or surveys (Campbell et al., 1999; De Jong et al., 2008). So far, the focus in such experiments was only on the active component of zooplankton communities, and even though natural sediment is often included as substrate, effects of pollutants on dormant egg bank dynamics are typically not taken into account. Therefore, we performed a two year outdoor mesocosm experiment, assessing impacts of repeated carbaryl exposure on both the active and dormant component of zooplankton communities (**chapter 5**). In addition, to assess whether effects on the dormant community would lead to changes in the active community and vice versa, also a dormant egg bank treatment was included in our experiment. This allowed us to test specifically for effects of pesticide exposure on newly produced dormant eggs as well as on dormant eggs already present in the sediment fraction (used to assess the buffering capacity of the egg bank). We observed significant negative effects of pesticide exposure on taxon richness and abundances of the established active communities. This was however not reflected in the composition of the dormant egg bank as we did not find any effects on newly produced dormant eggs or on the dormant eggs already present in the sediment fraction since the start of the experiment. This indicates that carbaryl had no direct or indirect effects on dormant egg bank dynamics. Since this was only a first study specifically designed to assess effects on dormant communities and the benthic-pelagic coupling, it would be interesting to test other exposure regimes and different model pesticides.

Hanazato (1998) observed that the recovery potential of the active phase was, amongst others, depending on the timing of exposure. Zooplankton communities could re-establish relatively rapidly when exposed in summer, but not after carbaryl application in spring or fall. Also, pesticide application later in the growing season might cause effects on dormant egg production, as observed for other pesticides under laboratory conditions (Olmstead and LeBlanc, 2001; Saika et al., 2006; **chapter 4**). We did not observe effects on newly produced dormant eggs in the current experiment, most likely because zooplankton populations had sufficient time to recover before the typical peak of sexual reproduction in fall. In addition, carbaryl showed no direct effects on hatching up to concentrations 1000 times the EC_{50} for *D. magna* neonate toxicity (EFSA, 2006; Coors et al., 2009) in laboratory hatching experiments (**chapter 1 – 2**). Other pesticides, such as fenoxycarb, that do show effects on hatching and production of dormant eggs under laboratory conditions (**chapter 1 - 4**) might also have a significant impact on the production, viability, or hatching process of dormant eggs in (semi-)natural systems. It would therefore be interesting to test this model pesticide on additional zooplankton species, as well as on the community level.

Future perspectives

With this thesis we aimed to get more insight into the effects of pesticide exposure on dormant egg bank quantity and quality. We did this by studying different pathways of impact of selected model pesticides on the dormant phase, ranging from 10-day laboratory experiments at population level to a 2-year outdoor mesocosm study at zooplankton community level. From our findings we suggest three approaches, at different levels of biological organization, for future research:

- First of all, at the population level, there is hardly any experimental data available on the effects of toxicants on dormant life stages when in a state of dormancy (diapause or quiescence). Results from **chapter 3** give a first indication that dormant eggs can also be affected before they are triggered by light to hatch. It would be interesting to further explore this scenario. A possibility would be to develop a method that could directly assess the viability of dormant eggs, without the need to perform hatching experiments. Gorokhova (2010) already applied a direct staining method to assess the viability of different zooplankton eggs, including parthenogenetic eggs of *D. magna*. In current studies, hatching of (previously) exposed dormant eggs was used as a proxy for the viability of dormant eggs. However, not all dormant eggs respond similarly to hatching cues (Vandekerkhove et al., 2004) and even under optimal conditions generally not all eggs will hatch as part of a risk spreading strategy (bet-hedging theory; Cohen, 1966; Evans and Dennehy, 2005), which might compromise the results of this type of experiments. If this staining method could be adapted for use in dormant zooplankton eggs, this could aid assessment of direct impacts of toxicants on dormant life stages.

- Secondly, laboratory microcosm bioassays for testing effects of pollutants on dormant community dynamics could be explored as an additional level in between single species laboratory studies and outdoor mesocosm experiments. These bioassays take into account species interactions and indirect effects while allowing for a higher level of repeatability, replicability and standardization than outdoor mesocosms (Walker, 2014). Clément et al. (2014) have developed a 2 L batch or flow-through water-sediment system containing an assembled community consisting of five species: *Pseudokirchneriella subcapitata* (green algae), *Lemna minor* (duckweed), *D. magna* (waterflea), *Hyalella azteca* (amphipods), *Chironomus riparius* (blood worm). They have used this system to test for potential toxic effects of contaminated sediments (Triffault-Bouchet et al., 2005; Clément et al., 2014). Test systems like this could be adapted and used to test specific scenarios regarding impact of pollution on dormant egg bank dynamics (Angeler and Garcia, 2005).

- And third, on a landscape level, it would be interesting to explore the effects of pollutants on dormant egg banks in natural systems. For example, sediment from pristine and polluted ponds could be sampled and used in subsequent laboratory hatching experiments, to assess differences in abundances and taxon richness, using methods similar to Angeler et al. (2005) and Henri et al. (2014). In addition, hatchling survival and performance could be tested in a life-table approach to assess chronic effects of pollution.

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SUMMARY

Currently, agricultural land comprises about 40% of the worlds land surface, producing food for six billion people. Projections are that the global human population will continue to increase and a doubling is expected by 2050. This continued population growth is accompanied by a sharp increase in food demand, which has put a lot of pressure on the agricultural sector to increase crop yields. Pesticides have been increasingly used to help boost crop production. By spray drift, run-off and leaching, a fraction of these pesticides ends up in aquatic water bodies in or surrounding agricultural areas, thereby potentially affecting also non-target species, such as planktonic organisms. Many zooplankton taxa depend on dormant life stages to survive unfavourable environmental conditions (drought, freezing, predation). To date however, not much is known about the effects of pollution on dormant life stages. Toxicants could have an impact on both the active and dormant phase of zooplankton populations and communities, through the following scenario´s: 1) impact on development and hatching of dormant eggs; 2) effects on hatchling survival and performance in the aquatic phase; 3) impact on dormant stages before activation (in diapause), causing egg mortality or irreversible disruption of the dormancy-break system; 4) effects during the sexual reproductive phase, affecting dormant egg production.

To test whether pesticides can be used safely, ecological risk assessments are performed, of which ecotoxicological assays form an important part. While aquatic invertebrates are routinely tested in ecotoxicological studies (especially the model organism *Daphnia magna*), most of these studies focus on a small part of their life cycle: asexual reproduction of clonal lineages (scenario 2). Studies investigating effects of pollutants on dormant life stages and the sexual reproductive phase are vastly underrepresented (scenario 1, 3 and 4). With this doctoral thesis we aimed to improve our understanding of the effects of pesticide exposure on dormant egg bank dynamics. Therefore, we studied different endpoints related to the dormant phase in zooplankton populations and communities, both under controlled conditions in the laboratory, as well as in a semi natural mesocosm environment. An important goal was to identify which part of the life-cycle in the model organism *D. magna* was most sensitive to toxicant exposure. In addition, we explored what new information regarding the sensitivity and recovery potential of aquatic communities could be obtained from including effects on dormant egg bank dynamics in higher tier ecotoxicological studies.

At the population level we performed a series of integrated laboratory experiments using *D. magna*. Our results indicate that, in addition to inducing mortality of active individuals (scenario 2), pesticides can also affect hatching dynamics and life history traits of hatched individuals (scenario 1). We tested five different model pesticides for their effects on both dormant (sexual) and parthenogenetic (asexual) eggs of *D. magna*. The effects on dormant life stages differed among toxicants, depending on their mode of action and potential for bioaccumulation. Even a pesticide such as carbaryl that had no direct effect on the hatching process, still caused negative chronic effects on survival and hatchling performance. The impact of pesticide exposure was not only determined by the type of toxicant, but also by the timing of exposure. Final stages of embryonic development were most sensitive to pesticide exposure and had the highest measured internal pesticide concentrations.

Even before light activation, dormant eggs could already be affected by toxicant exposure (scenario 3). In addition, the ephippial case that surrounds dormant eggs under natural conditions, offered limited or no direct protection against pesticide exposure. This indicates that even though dormant eggs show a high tolerance to extreme physical conditions, they can still be affected by chemical pollution. It remains to be tested whether the effects of pollutants on dormant eggs are also depending on the type of dormancy (i.e. quiescence versus diapause).

In our research we showed that exposure to the juvenile growth hormone fenoxycarb could also affect the sexual reproductive phase in *D. magna* (scenario 4). Fenoxycarb caused a decrease in both parthenogenetic and dormant egg production, while inducing the production of male offspring. There were no significant effects of fenoxycarb exposure on the survival and life history characteristics of the hatchlings, when exposure took place during dormant egg production. This indicates that even though the quantity of the dormant eggs was reduced, their quality was not affected significantly by fenoxycarb exposure.

To assess the effects of pesticide exposure at the zooplankton community level, we performed a two year outdoor mesocosm experiment, focusing on impacts of repeated carbaryl exposure on both the active and dormant phase. The inclusion of a dormant egg bank treatment allowed us to test specifically for effects of pesticide exposure on newly produced dormant eggs as well as on dormant eggs already present in the sediment fraction (used to assess the buffering capacity of the egg bank). The active communities were negatively affected by carbaryl exposure, especially the smaller sized cladocerans (*Ceriodaphnia quadrangula en Chydorus sphaericus).* We did not observe effects on newly produced dormant eggs in the current experiment, most likely because zooplankton populations had sufficient time to recover before the typical peak of sexual reproduction in fall. Hatching of dormant eggs already present in the sediment was not affected by carbaryl exposure. This is in agreement with results from our laboratory experiments, were carbaryl showed no direct effects on hatching up to concentrations 1000 times the effect level (EC_{50}) for *D. magna* neonates.

Our results clearly indicate that pollution can affect dormant egg bank dynamics in zooplankton populations through all scenarios evaluated (1-4). In general, the effect levels found in our laboratory experiments are not lower than those obtained by standard ecotoxicological screening assays. We proved that pesticides can have long-term effects on dormant egg bank dynamics, which could have important consequences for ecological and evolutionary dynamics of zooplankton populations and communities in lakes and ponds. More research is needed to determine the long-term ecological impact of exposure to environmental relevant concentrations of pesticides, under different exposure scenarios, in (semi-)natural aquatic ecosystems. Laboratory microcosm bioassays for testing effects of pollutants on dormant community dynamics could be explored, as an additional level in between single species laboratory studies and outdoor mesocosm experiments. And finally, it would be interesting to explore the effects of pollutants on dormant egg banks in natural systems, by studying impacted versus pristine sites.

SAMENVATTING

Momenteel bestaat ongeveer 40% van het aardoppervlakte uit agrarisch landschap, waarop voedsel voor 6 biljoen mensen wordt geproduceerd. De verwachting is dat de wereldbevolking verder zal blijven stijgen en tegen 2050 opnieuw zal zijn verdubbeld. Dit zal samengaan met een alsmaar groter wordende vraag naar voedsel. Om de productiviteit te blijven verhogen worden onder andere meststoffen en pesticiden gebruikt en nieuwe gewassen ontwikkeld. Een deel van de gebruikte pesticiden komt door overwaaien bij het spuiten, uitloging of wegspoeling terecht in oppervlaktewateren, zoals vijvers en meren, die in de omgeving van akkers en weilanden liggen. Hierdoor kunnen ook niet-doelorganismen, zoals zooplankton soorten, blootgesteld worden aan pesticiden. Veel van deze zooplankton soorten zijn in hun voortbestaan afhankelijk van dormante levensstadia om ongunstige perioden (droogte, vrieskou, predatie) te overbruggen. Er is echter weinig bekend over de effecten van pollutie op dormante levensstadia. Toxicanten kunnen een invloed hebben op zowel de actieve als de dormante fase van zooplankton populaties en gemeenschappen volgens verschillende scenario's: 1) impact op de ontwikkeling en ontluiking van dormante eieren; 2) effecten op de overleving en performantie van ontloken individuen in de actieve fase; 3) impact op dormante diapauserende stadia waardoor dormante eieren afsterven of hun ontluikingsmechanisme onherstelbaar beschadigd wordt; 4) effecten tijdens de sexuele reproductieve fase, waardoor de productie van dormante eieren aangetast wordt.

Om te testen of pesticiden veilig gebruikt kunnen worden, worden ecologische risico-evaluaties uitgevoerd. Ecotoxicologische testen vormen hier een belangrijk onderdeel van. Standaard ecotoxicologische testen op zooplankton soorten zijn op dit moment vooral toegespitst op de effecten van toxicanten op overleving en reproductie van organismen in de actieve fase (scenario 2), over de effecten op scenario´s 1, 3 en 4 is veel minder bekend. Met deze doctoraatsthesis wilden we meer inzicht verkrijgen in de effecten van blootstelling aan pesticiden op dormante rusteierenbank dynamieken. Daarvoor hebben we verschillende eindpunten, gerelateerd aan de dormante fase van zooplankton populaties en gemeenschappen bestudeerd, zowel onder gecontroleerde laboratorium condities als in semi-natuurlijke test systemen (mesocosms). Een belangrijke doel was om te achterhalen welk deel van de levenscyclus van het model organisme *Daphnia magna* (watervlo) het meest gevoelig was aan pesticiden blootstelling. Daarnaast hebben we onderzocht wat voor nieuwe informatie over de gevoeligheid van zooplankton gemeenschappen we konden verkrijgen door de effecten van pesticiden specifiek op dormante rusteierenbank dynamieken te testen, in een mesocosm experiment.

Op populatie niveau hebben we een serie van geïntegreerde laboratorium experimenten uitgevoerd met *D. magna*. Onze resultaten geven aan dat, pesticiden niet alleen mortaliteit in de actieve gemeenschap kunnen veroorzaken (scenario 2), maar ook effecten kunnen hebben op ontluikingsdynamieken en levensgeschiedeniskenmerken van ontloken individuen (scenario 1). We hebben een vijftal pesticiden getest op zowel dormante (sexuele) als parthenogenetische (asexuele) eieren van *D. magna*. De effecten van de geteste toxicanten op dormante levensstadia verschilden tussen de pesticiden, afhankelijk van hun werkingsmechanisme en potentieel voor bioaccumulatie.

Zelfs een pesticide als carbaryl dat geen direct effect had op het ontluikingsproces, veroorzaakte toch negatieve chronische effecten op overleving en performantie van ontloken organismen. De impact van de pesticide blootstelling werd echter niet alleen bepaald door het type toxicant, maar ook door het tijdstip van toediening. De laatste stadia van embryonale ontwikkeling waren het meest gevoelig aan pesticiden blootstelling. Hier vonden we ook de hoogste interne concentraties van pesticiden gemeten in de dormante eieren terug. Zelfs nog voordat de dormante eieren geactiveerd waren om te ontluiken, konden ze al aangetast worden door pesticiden blootstelling (scenario 3). Daarnaast bleek het ephippium (beschermende kapsel) rond de dormante eieren, nauwelijks of geen bescherming te bieden tegen pesticiden blootstelling. Dit geeft aan dat ook al hebben dormante eieren een hoge tolerantie voor extreme fysische condities, zoals vrieskou of langdurige droogte, ze toch een negatieve invloed kunnen ondervinden van chemische vervuiling. Verder onderzoek zal moeten uitwijzen of de effecten van vervuiling ook verschillen tussen de uiteenlopende stadia van dormantie (quiescentie versus diapause).

We hebben in ons onderzoek aangetoond dat blootstelling aan de insect groeiregulator fenoxycarb in *Daphnia* ook een effect kan hebben op de sexuele reproductieve fase (scenario 4). Fenoxycarb veroorzaakte een daling in zowel de productie van parthenogenetische eieren als dormante eieren, en induceerde de productie van mannetjes. We hebben geen significante effecten gevonden van fenoxycarb blootstelling op overleving en levensgeschiedeniskenmerken van ontloken individuen, wanneer de dormante eieren waren blootgesteld tijdens de productie. Dit geeft aan dat alhoewel de hoeveelheid geproduceerde dormante eieren significant verminderde, de kwaliteit van de dormante eieren in dit scenario niet negatief beïnvloed werd door fenoxycarb.

In een tweejarig mesocosm experiment hebben we onderzoek gedaan naar de effecten van pesticiden blootstelling op zooplankton gemeenschappen, toegespitst op de impact van herhaalde carbaryl toediening op zowel de actieve als dormante fase. Toevoeging van een dormante eierenbank behandeling in ons experiment, heeft er voor gezorgd dat we specifiek konden testen voor de effecten van pesticide blootstelling op zowel nieuw geproduceerde dormante eieren, als op dormante eieren die al aanwezig waren in de sediment fractie vanaf de start van het experiment. De actieve gemeenschappen werden negatief beïnvloed door de toediening van carbaryl. Vooral kleinere zooplankton taxa (*Ceriodaphnia quadrangula* en *Chydorus sphaericus*) waren gevoelig voor de pesticiden blootstelling. We hebben geen effecten gevonden van carbaryl blootstelling op nieuw geproduceerde rusteieren, waarschijnlijk omdat de actieve zooplankton populaties voldoende tijd hadden om te herstellen van de blootstelling, voordat de piek van de rusteieren productie plaatsvond. De ontluiking van rusteieren die al aanwezig waren in het sediment, werd niet negatief beïnvloed door carbaryl bloostelling. Dit is in overeenstemming met resultaten bekomen in de laboratorium experimenten, waarbij carbaryl geen directe effecten had op ontluiking tot concentraties 1000 keer hoger dan de effect niveaus (EC_{50}) voor *D. magna* neonaten.

Onze resultaten tonen duidelijk aan dat chemische vervuiling een effect kan hebben op dormante rusteierenbank dynamieken in zooplankton populaties, via alle onderzochte scenario´s (1 - 4). Over het algemeen zijn de gevonden effect niveau´s in onze laboratorium experimenten niet lager dan effect niveau´s bekomen in standaard ecotoxicologische screening testen. We hebben wel aangetoond dat pesticiden langdurige effecten kunnen hebben op rusteierenbank dynamieken en dit kan een belangrijke impact hebben op evolutionaire en ecologische dynamieken van zooplankton populaties en gemeenschappen. Verder onderzoek is nodig om uit te wijzen wat de lange-termijn effecten van bloostelling aan milieu relevante pesticiden concentraties, via verschillende blootstellingsscenario´s, zijn op zooplankton gemeenschappen in natuurlijke aquatische systemen. Daarnaast zouden laboratorium microcosms gebruikt kunnen worden om de effecten van polluenten te testen op dormante rusteierenbank dynamieken, als schakel tussen laboratorium experimenten met één soort en openlucht mesocosm studies op gemeenschapsniveau. Als laatste, zou het interessant zijn om de effecten van chemische vervuiling te testen op dormante eierenbanken in natuurlijke systemen, door het bestuderen van geïmpacteerde en pristiene locaties.